ARTERIAL IN VITRO REMODELING:
ANALYSIS BIOMECHANICAL AND BIOLOGICAL FACTORS
INFLUENCING THE ADAPTIVE RESPONSE

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SUMMARY

Vascular remodeling is defined as any enduring change in the size and composition of an adult blood vessel, allowing adaptation or repair. The vascular remodeling response has been shown to depend on a variety of endogenous and environmental factors. Physiological remodeling is a tightly regulated process that mainly occurs in response to long-term changes in hemodynamic conditions. The adaptation to these hemodynamic changes implies the production of mediators that influence structure as well as function. A loss of regulation in the adaptive response underlies the pathogenesis of major cardiovascular diseases, including hypertension, atherosclerosis, restenosis and arterial aneurismal dilatation. Specialized enzymes called matrix metalloproteinases (MMPs) have been shown to have a predominant participation in the reorganization of the vessel structure, through the degradation of the extracellular matrix scaffold.

The aim of this thesis is to gain insight in the biological and mechanical processes taking place in the vascular wall as an adaptive response to different biomechanical stimuli such as blood pressure and blood flow. This work proposes a new model for the study of vascular remodeling where physical factors acting on the arterial wall can be dissociated and analyzed, individually, in relation to the biological response. The results are presented in form of an introduction, three scientific papers and a conclusion section.

The investigation has been designed around three different approaches: adaptation of a non-uniform artery to an in vitro environment, vascular adaptation to steady and pulsatile pressure and vascular adaptation to unidirectional and oscillatory flow. The adaptive response to each one of the variables chosen has been analyzed through biological and biomechanical remodeling indicators of the arterial wall.

The introduction is an overview of the biological and biomechanical characteristics of arterial wall in relation to the remodeling process. The contribution of vascular smooth muscle cells and extracellular matrix to physiological and pathological arterial remodeling is discussed.
Paper I assesses the relative remodeling of a non-axisymmetric artery in relation to its environment. The study considers the native circumferential asymmetry of the porcine right common carotid, which results from the non-homogenous mechanical and hemodynamic native environment. The adaptive response of the artery to an in vitro perfusion environment is analyzed. The study shows that in vitro perfusion leads, through remodeling, to a circumferentially uniform scleroprotein distribution and to a change in arterial compliance. This study emphasizes the link between structural changes, the biomechanical response and the enzymatic implication in the adaptive response.

In paper II we investigate the role of continuous and cyclic stretch, produced by steady or pulsatile pressure acting on the arterial wall, on the remodeling response. The study shows that exposure to continuous and cyclic stretch differentially affects the relative scleroprotein content and leads to a change in arterial wall stiffness. The adaptive outcome is studied through an integrative approach taking into consideration the geometrical and structural adaptation, the biomechanical behavior and the enzymatic agents implicated in extracellular matrix turnover.

Paper III analyzes the influence of different flow patterns on the arterial adaptive response. We investigate the differential effects of oscillatory flow, mimicking a plaque-prone hemodynamic environment, and unidirectional flow, to mimicking a physiologically protective (plaque-free) environment. The effect of these hemodynamic forces on the remodeling response are characterized through the study of endothelial and smooth muscle cell function as well as through assessment of agents influencing extracellular matrix turnover.

The conclusions section presents a synthesis of the results and contribution of this thesis and proposes perspectives for future studies.
Le remodelage vasculaire se définit comme tout changement durable dans la taille et composition d’un vaisseau sanguin adulte, permettant ainsi une adaptation ou/et une réparation. Le processus de remodelage vasculaire dépend d’une variété de facteurs endogènes et environnementaux. Le remodelage physiologique est un processus adaptatif finement contrôlé qui répond aux changements, à long terme, des conditions hémodynamiques. Cette adaptation implique la production de médiateurs qui influencent la structure et la fonction du vaisseau. Une perte de regulation du processus adaptatif est sous-jacente à la pathogenèse des principales maladies cardiovasculaires. Des enzymes spécialisées, dénommées métalloprotéases de la matrice (MMPs), ont une participation prédominante dans la réorganisation de la structure vasculaire. Ces enzymes dégradent l’échafaudage de la paroi constitué par la matrice extracellulaire.

L’objectif de cette thèse est d’acquérir une connaissance plus approfondie des processus biologiques et mécaniques impliqués dans la réponse adaptative des artères aux différents stimuli biomécaniques tels que la pression ou le débit sanguin. Ce travail propose un nouveau modèle pour l’étude du remodelage vasculaire permettant la dissociation des facteurs physiques agissants sur la paroi artérielle et leur analyse individuelle en relation à la réponse biologique observée. Ce travail de thèse se compose d’une introduction, de trois articles scientifiques soumis à publication ou publiés et d’une conclusion.

Le travail de recherche a été organisé selon trois différentes approches : adaptation d’une artère non uniforme à un environnement *in vitro*, adaptation artérielle à une pression statique et à une pression pulsatile, et adaptation artérielle à un débit unidirectionnel versus un débit oscillatoire. La réponse adaptative de la paroi artérielle à chacune des variables choisies a été analysée en utilisant des indicateurs biologiques et biomécaniques de remodelage.

L’introduction résume l’état des connaissances actuelles des caractéristiques biologiques et biomécaniques de la paroi artérielle au cours du processus de remodelage. En particulier, la contribution des
cellules musculaires lisses et de la matrice extracellulaire aux remodelages physiologique et pathologique est discutée.

Le premier article évalue le remodelage relatif d’une artère non-axisymétrique en relation à son environnement. Cette étude considère l’asymétrie native de la circonférence de la carotide commune droite de porc comme le résultat d’un environnement mécanique et hémodynamique natif non-homogène. L’étude a pour but d’analyser l’adaptation artérielle à un environnement de perfusion in vitro. Ce travail de recherche démontre que la perfusion in vitro conduit, par un processus de remodelage, à une distribution uniforme de scléroprotéines dans le sens de la circonférence de l’artère, et à un changement de compliance artérielle. Cette étude souligne le lien existant entre les changements structurels, la réponse biomécanique et l’implication enzymatique dans l’adaptation artérielle.

Dans le deuxième article, la réponse adaptative de la paroi artérielle à l’étirement continu, produit par une pression statique, et à l’étirement cyclique, produit par une pression pulsatile, est étudiée. Cette étude montre que le contenu relatif de scléroprotéines et la rigidité de la paroi artérielle varient de manière différente si l’artère est exposée à une pression statique ou à une pression pulsatile. Le remodelage est évalué selon une approche intégrative qui tient compte de l’adaptation géométrique et structurelle de la paroi ainsi que de son comportement biomécanique et des agents enzymatiques impliqués dans la dégradation de la matrice extracellulaire.

Le troisième article analyse l’influence de différents paramètres de débit sur la paroi artérielle. Dans ce travail, les effets différentiels d’un débit oscillatoire, qui reproduit un environnement hémodynamique favorable au développement de plaques athérosclérotiques, et d’un débit unidirectionnel qui reproduit un environnement non favorable au développement de plaques (environnement protecteur), sont étudiés. La réponse adaptative est évaluée à travers la caractérisation de la fonction des cellules endothéliales et des cellules musculaires lisses ainsi que des agents moléculaires influençant la matrice extracellulaire.

Le dernier chapitre, en conclusion, résume les résultats obtenus, définit la contribution de ce travail et propose des perspectives pour des études futures.
Making a thesis in vascular remodeling was a most valuable experience to live. The difficult and rewarding moments that I found on the way significantly contributed to my personal and professional development. Vascular remodeling is a complex field which understanding can only be approached by pulling together concepts and methodologies from a variety of disciplines. Doing research in an interdisciplinary field is a challenge to overcome, as its progress relies on strategic decisions taken under conditions of considerable uncertainty. Living interdisciplinary research is also very enriching, as the daily contact between several disciplines is an opportunity to go beyond our own limits.

I express my gratitude to Professor Nikos Stergiopulos for offering me the opportunity of living the experience of this thesis, for sharing his wide knowledge and enthusiasm on this vast subject and for opening me the doors of his scientific environment.

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This thesis work points at the important issue of the role of mechanical stress in tissue homeostasis. Specifically, it addresses the relevance of the interrelation between hemodynamic changes and tissue constituents in physiological and pathological remodeling.

The aim of this study is to gain insight in the biological and mechanical processes taking place in the vascular wall as an adaptive response to local hemodynamic changes involving tensile stress, related to blood pressure, or shear stress, related to blood flow. The study is directed towards an integrative approach that considers the biomechanical response, wall structural changes and the enzymatic mechanisms acting in the remodeling process.
INTRODUCTION
ARTERIAL REMODELING

Adequate function of the arterial tree depends on the maintenance of lumen patency and blood pressure levels, which assure continuous blood flow to the organs and tissues of the body. During organ growth and development, arterial cells differentiate and associate into a set of vessels of precisely the appropriate length, diameter, structure and composition in relation to their localization and function. The vasculature is capable of sensing changes within its hemodynamic and metabolic environment, integrating these signals by intercellular communication, and adapting its geometry, structure and function through the local production of biological mediators. Metabolic or physical stresses may, in some cases, result in cell dysfunction and necrosis or tissue disruption and disorganization which can trigger a healing processes tending to preserve adequate function or at least to postpone total loss of integrity. However, changes in mural structure and composition which at one stage seem to be adaptive may in long-term cause alterations in the cell-stimuli coupling response, and lead ultimately to irreversible, destructive processes.

Structural adaptive responses of the artery wall are normally elicited by two principles mechanical factors, wall shear stress and tensile stress. Wall shear stress is a frictional deforming force at the blood-endothelium interface that depends on the gradient of near-wall blood flow velocities. The magnitude of wall shear stress is related directly to flow rate and blood viscosity and inversely to the third power of the vessel radius. Changes in flow engender changes in radius that stabilize when baseline wall shear stress is restored. Wall tension, the force tending to stretch the wall, is determined by distending pressure and also by the radius. Thus, a change in radius in response to a change in wall shear stress results in a change in wall tension. Corresponding modifications in thickness and composition assure stability of the wall. The term remodeling summarizes the results of these putative compensatory processes at the tissue level.

REMODELING OF ELASTIC ARTERIES

During growth and development vascular geometry and structure adapt to immediate and chronic physical and metabolic variations. Cell
dysfunction or tissue disruption and disorganization due to changes in the hemodynamic and metabolic environment induce adaptive or healing processes, known as vascular remodeling, tending to preserve adequate function or delay the irreversible degradation of the organ.

From a biological perspective vascular remodeling can be defined as an active process of structural alteration that involves changes in at least four cellular processes –cell growth, cell death, cell migration and production of extracellular matrix- and is dependent on a dynamic interaction between growth factors, vasoactive substances and hemodynamic stimuli [1].

**Structural properties of the arterial wall**

Histologically, the arterial wall is composed by three tissue layers, called from the luminal side outward, the tunica intima, the tunica media and the tunica adventitia (fig. 1). The thickness of these three layers varies greatly depending upon the size and type of the vessel: elastic or conduit arteries, medium or muscular arteries and small arteries or arterioles. These types of vessels are all continuous with one another.
Figure 1: Histological structure of the arterial wall (SMC: smooth muscle cells, ECM: extracellular matrix)

**Elastic arteries**

The aorta and its branches (brachiocephalic, subclavian, pulmonary, beginning of common carotid and iliac) are distinguished by their great elasticity. This characteristic contributes to smooth out the large fluctuations in blood pressure created by the heartbeat.

The tunica intima consists of a monolayer of endothelial cells connected to each other by tight junctions and sustained by a basal lamina composed by collagen and elastin fibers, smooth muscle cells and occasional fibroblasts. The border of the intima is delineated by the internal elastic lamina, which may not be conspicuous in conduit arteries because of the abundance of elastic material in the tunica media. The endothelium serves as a sensory cell assessing hemodynamic and humoral signals, as well as an effector cell eliciting biologic responses. The homeostatic balance is maintained by the release of endogenous vasoactive substances (vasoconstrictive as well as vasodilative),
procoagulants and anticoagulants, proinflammatory and anti-inflammatory mediators, growth promoters and growth inhibitors [1]. The structural properties of the endothelium at both cellular and ultrastructural levels are highly sensitive to shear stress and rapid remodeling of these cells can occur when shear changes [2]. Endothelial cells can participate directly in vascular remodeling by releasing or activating substances that influence the growth, death and migration of cellular elements or the composition of extracellular matrix. These cellular and extracellular components interact closely, and their relation ultimately determines the geometry of the blood vessel [1].

The tunica media is the thickest of the three layers. Smooth muscle cells, that under normal circumstances constitute the single medial cell type, are arranged in spiral around the long axis of the vessel and connected to each other via gap junctions. They are capable of synthesizing and secreting all the principal components of the extracellular matrix and therefore are responsible for the formation of elastic lamellae, which are fenestrated to facilitate diffusion, as well as of collagen and proteoglycans that surround each lamella. As a consequence of their ability to secrete all extracellular matrix components, they play a main role in the maintenance of medial architecture and remodeling. Vascular smooth muscle cells perform several functions in the blood vessel wall according to their specific location and presence or absence of disease. In normal vessels, smooth muscle cells have a contractile phenotype and their ability to develop maximal force is equal or greater than that of striated muscle [3], they are capable of responding to vasoactive agents that can modulate cell contractility and thus alter vascular tone.

The tunica adventitia is a relatively thin connective tissue layer. Fibroblasts are the predominant cell type, and many macrophages are also present. Collagen fibers are in greater proportion than elastic fibers. Blood vessels (vasa vasorum) supply the adventitia and outer media; the inner part of the media is supplied from the lumen via pinocytic transport.
Muscular arteries

There is no sharp dividing line between elastic and muscular arteries; in areas of transition, arteries may appear as intermediate between the two types.

The tunica intima is thinner than in large arteries, there are fewer smooth muscle cells and less elastic tissue. A very prominent internal elastic membrane defines the outermost part of the intima. The basement membrane of the endothelium may rest directly on the internal elastic membrane, or be separated by a subendothelial layer of connective tissue.

In the tunica media smooth muscle is the predominant constituent and little elastic material is present. As in large arteries, no fibroblasts are present.

The tunica adventitia is relatively larger than in elastic arteries, it can be up to the same size as the media and it often blends with the connective tissue of the surrounding structures. The main constituent of the tunica adventitia is collagen fibers, secreted by fibroblasts. Elastic fibers are also present; a concentration of such fibers at the inner boundary of the adventitia is called the external elastic membrane. The external elastic membrane is not as prominent as the internal, and as arteries get smaller it disappears.

Small arteries

The general construction of small arteries is very similar to that of muscular arteries. The intima becomes smaller and the internal elastic membrane eventually disappears. The media is still muscular and has up to 8-10 layers of smooth muscle cells. This number is reduced as the arteries get smaller; the smallest arterioles have 1-2 layers of smooth muscle cells. The adventitia becomes thinner and the external elastic membrane is absent.

Mechanical properties of the arterial wall

The distinct structural elements that compose the arterial wall function in a coordinated manner to assure stability and resiliency in relation to
imposed mechanical forces. The mechanical characteristics of large vessels have been attributed to the properties of the scleroproteins present in the wall, in particular to elastin and collagen. Elastin is a fibrous, elastomeric protein capable of extending more than 100%, while collagen can be stretch only 3-4% [4]. In conduit arteries, collagen fibers normally bear a major portion of the tensile stress, and when drawn taut, prevent excessive distension. The extensible elastin network contributes to tensile support but also provides resiliency by distributing stresses uniformly through the wall. In this manner, disruptive propagation of structural flaws is prevented and a graded mobilization of the media is assured [5].

*Pressure-diameter and pressure-thickness relations*

Cylindrical wall tension as defined by Laplace is the product of pressure and radius. At low values of wall strain, wall tension is primarily absorbed by elastin fibers [6, 7]. As strain increases, collagen fibers are sequentially recruited [6, 7]. Since collagen fibers are more rigid than elastin fibers, as the intraluminal pressure increases the lumen diameter enlarges in a non-linear behavior, resulting in a sigmoid diameter-pressure curve (fig. 2) [6, 7]. As the arterial circumference is increased the arterial wall thickness is decreased, as dictated by the incompressibility of the arterial wall (fig. 2) [8].
Compliance and distensibility

The buffering capacity of an arterial system is termed compliance and is determined by the local slope of the pressure-volume relation. For a small change around a chosen point the pressure-volume curve is approximately straight and can be represented by its tangent. The slope of the tangent, thus compliance, can be calculated as:

*Figure 2: Pressure-diameter and pressure-thickness relations measured for an elastic artery*
where $C$ is compliance, $V$ is volume and $P$ is pressure (fig. 3). If instead of an entire organ, a cross-sectional area is measured, as it is often done in blood vessels, then area $A$ replaces volume $V$.

Figure 3: The pressure-volume relation at a given point of the curve can be represented by the tangent to this point. The slope of the tangent determines Compliance ($C$).

Compliance can be measured at systemic or local level. Systemic compliance is an index for the compliance of the entire arterial system while local compliance is measured at the level of a given artery. In
other words, systemic arterial compliance is the summation of local compliances of all the arteries that contribute to the buffering function of the vascular system. The stiffer the arteries, the lower the systemic arterial compliance, which reflects a low total buffering capacity of the vascular system. The ratio of stroke volume to pulse pressure is generally used as an indication of systemic compliance [9].

The buffering capacity of an artery or its compliance is determined by the arterial distensibility and the arterial lumen volume. The arterial lumen volume is determined by the arterial length and diameter. The arterial distensibility is the relative change in volume for a given increase in pressure and is calculated as:

\[ D = \frac{1}{V} \frac{\partial V}{\partial P} \]

where \( D \) is distensibility, \( V \) is volume and \( P \) is pressure. If a cross-sectional area is measured, then area \( A \) replaces volume \( V \).

Distensibility gives an indication of the behavior of the arterial wall as determined by its wall components and their arrangements. At low pressures and small diameters the arteries are very distensible, whereas with increasing pressure and diameter they become gradually stiffer. This is manifest in the shape of curves describing distensibility-pressure relations, where a gradual transition from compliant to stiff behavior can be observed in normal large arteries (fig. 4) [4].
**Introduction**

**Wall elasticity**

Vascular wall components as well as the geometry of their arrangement are important in influencing the mechanical properties of the artery. Distensibility describes the structural behavior of the arterial wall in relation to its components and their configuration, and thus it does not characterize the elastic properties of the material itself. Material properties are described by the incremental elastic modulus, which represents a measure of the material stiffness. Therefore, although the distensibility of an artery may be reduced due to an increase in wall thickness, the incremental elastic modulus may not be altered indicating that the change in distensibility is not due to a change in the nature of the vascular wall components but solely to an increase in its mass.

In general, stiffness is the force required to impose a certain degree of deformation upon a body. The higher the force needed and the smaller the resulting deformation, the higher the stiffness. When this definition is applied to large arteries, deformation in the radial direction is an increase in diameter, which results in an increase in luminal cross-sectional area. In practice, the incremental elastic modulus can be calculated from pressure-diameter relations as:
where \( r_i \) is the internal radius, \( r_o \) is the external radius and \( P \) is pressure (fig. 5).

\[
E_{inc} = \frac{3}{2} \frac{r_i^2 r_o}{r_o^2 - r_i^2} \frac{dP}{dr_o}
\]

Figure 5: Incremental elastic modulus - circumferential stretch ratio relation measured for an elastic artery

Contribution of smooth muscle cells to the arterial wall mechanics

Smooth muscle cells are the contractile elements of the arterial wall; they provide active tensile support and control vascular tone. Smooth muscle can be stimulated to contract or relax by multiple types of signals of mechanical or biochemical nature. Vasoactive substances produced by the endothelium such as nitric oxide can induce the muscle to relax in response to a sudden increase in blood flow, thus allowing the artery to restore shear stress values to normal levels [10]. When the smooth muscle is not stimulated it remains in a state of slight contraction known as basal vascular smooth muscle tone. Remaining in a state of slight contraction gives the artery the possibility to further relax
or contract without consuming excessive amounts of energy as required to maintain higher contraction levels. A sudden increase in intraluminal pressure may induce a myogenic response of the arterial wall. This mechanism is characterized by an abrupt contraction aiming to protect the arterial wall from excessive distension and is often observed in muscular arteries [11]. Experimental data on arterial pressure-diameter relation indicate that SMC contribute considerably to the stress sustained by the vessel wall [12]. As shown in figure 6, in arteries under normal tone at low pressures (0 kPa) SMC are in a relaxed state as evidenced by diameter values very similar to those measured for totally relaxed arteries. A step increase in pressure (25 kPa) induces non stimulated SMC (normal tone) to contract as evidenced by diameter values very similar to those measured for totally contracted arteries.

Vascular smooth muscle cells have an important role in the adaptive biosynthetic response of the artery. They are capable of secreting all extracellular matrix components and therefore assure the appropriate quantities and relative proportions of matrix components needed for stability and resiliency [13].

![Figure 6: Pressure-diameter relation as a function of muscular tone](image-url)
Arterial wall adaptation to stress variations

Arterial wall adaptation to tensile stress

Elastic arteries remodel their wall geometry and structure in response to changes in blood pressure. Few days of arterial exposure to pressure variations induce changes in tissue volume, reorganization of tissue structure, regulation of vascular smooth muscle tone, and adaptation of the vascular smooth muscle properties [14-16].

In arteries exposed to hypotension an increase in diameter is observed due to a reduction in vascular smooth muscle tone [17]. In response to increased arterial pressure, the vessel structure is altered such that the ratio of wall thickness to lumen diameter is elevated by either an increase in muscle mass or rearrangements of cellular and non cellular elements [18-20].

Figure 7 summarizes a terminology proposed by Mulvany et al. [21] that allows a precise description of the changes that are observed in the vasculature in response to pressure variations.
Introduction

Figure 7: Alterations in pressure induce a remodeling process that modifies the cross-section of blood vessels. Remodeling can be hypertrophic (e.g., doubling of cross-sectional area, vessels in right column), eutrophic (no change in cross-sectional area, vessels in center column), or hypotrophic (halving of cross-sectional area, vessels in left column). These forms can be inward (e.g., 30% reduction in lumen diameter, vessels in top row), or outward (e.g., 30% increase in lumen diameter, vessels in bottom row). From Mulvany et al. [21]

Eutrophic inward remodeling is observed in resistance vessels from essential hypertensive patients, where media-to-lumen ratio is increased and lumen is decreased without change in the amount of material [22]. Hypotrophic inward remodeling is observed in renal afferent arterioles, where lumen diameter is decreased with a decrease in the amount of material [23]. Hypertrophic inward remodeling of small arteries is associated with renal hypertension in rats [24]. The term Eutrophic outward remodeling designs the change observed in essential hypertensive patients following an antihypertensive therapy which causes an increase in lumen of small arteries without change in the amount of material [25]. Hypotrophic outward remodeling has been
observed in experiments with treatment of spontaneously hypertensive rats, where there was an increase in lumen of small arteries but a decrease in the amount of material [26]. Hypertrophic outward remodeling has been seen in the esophageal veins of rabbits following partial occlusion of the portal vein [27].

During essential hypertension, large-artery remodeling is characterized mainly by an increase in intima-media thickness (hypertrophy). The wall thickens to the point where it counterbalances the increase in pressure, thereby lowering wall hoop stress down to control levels [1] [28]. An example of such adaptation was described by Matsumoto et al. [29] who studied the effects of hypertension on stress and strain distributions through the wall thickness in the rat thoracic aorta. They showed that wall thickening caused by hypertension is mainly due to the hypertrophy of the lamellar units of the media, especially in the subintimal layer where the stress increase developed by hypertension is larger than in other layers (fig. 8).

![Figure 8: Thoracic rat aortas fixed under their in vivo pressure and stained with Azan. Systolic pressures were of 140 mmHg for control rats, 200 mmHg for hypertensive rats and 240 mmHg for severely hypertensive rats. Sections are parallel to the longitudinal axis of the vessel with the intimal surface facing leftward. From [29].](image-url)
Studies done on artificial vessels have demonstrated the important role of cyclic stress on metabolism and mechanical properties of the arterial wall, in particular the media [30, 31]. Kim et al. [32] demonstrated that short-term application of cyclic strain on engineered tissue increased smooth muscle cell proliferation and collagen and elastin expression while long-term application of cyclic strain upregulated elastin and collagen gene expression and led to increased tissue organization. Cyclic mechanical stress due to the pulsatile nature of blood pressure, has also been associated to large-artery remodeling [33, 34]. Carotid diameter enlargement has been shown to depend on heart rate and pulsatile pressure [35]. The remodeling response to stretch rate is attributed to fracture of the load-bearing elastin fibers in response to the fatiguing effect of tensile stress [36].

**Arterial wall adaptation to shear stress**

Vascular remodeling in response to flow alterations tends to restore normal baseline levels of wall shear stress. The adaptive process involves changes primarily in luminal dimensions and relatively small changes in wall thickness due to active restructuring of cellular and non cellular components [1]. When chronic alterations persist, then the diameter changes by remodeling medial tissues until the wall shear stress is restored to the normal level [37]. When an artery is enlarged in response to increased flow, the wall tensile stress is also increased. The intima may thicken to provide sufficient wall thickness to restore normal wall tensile stress [38]. Conversely, a long-term reduction in wall shear stress induces a decrease of the vascular mass and the lumen radius [1]. It appears that chronic adaptations to decreased flow proceed more rapidly than responses to increased flow. The rapid response to reduced flow probably occurs because diameter changes can be affected by smooth muscle cell contraction alone; thus a constriction of the vessel wall need only to be supported by relative subtle medial remodeling [39]. By contrast, early vasodilatory responses to increased flow are limited by the modest muscular tone usually exhibited by elastic arteries. A continuous process of vasodilatation and remodeling may be required to expand flow-loaded arteries [2]. Remodeling of arteries in response to chronically altered flow occurs in two phases, both endothelium dependent [40-42]. First, an increase of vascular tone is observed, then
the media remodels to alter the resting diameter of the artery [39]. A specific example of flow induced remodeling is given by the study published by Langille et al. [42] who reported that a 70 percent reduction in the blood flow rate of rabbit common carotid arteries caused a 21 percent diameter decrease of the artery within 2 weeks (fig. 9), and that this arterial response was abolished when the endothelium was removed.

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Figure 9: Scanning electron micrograph of methacrylate casts of left and right rabbit carotids under control (left) and 2 weeks after left carotid flow was surgically reduced (middle). Histological cross-sections of carotid arteries 2 weeks after left carotid flow was reduced (right). From [42].

In conduit arteries, the rapid vasodilatation that accompanies increased flow is mediated by the endothelium-derived vasoactive agent, nitric oxide (NO) [42]. Tronc et al. [43] investigated the role of endothelium-derived NO in the adaptive increase of arterial diameter in response to a chronic increase in blood-flow in rabbits. Chronic flow increase was induced by means of an arteriovenous fistula constructed between the carotid artery and the jugular vein in two groups of animals. One group was submitted to inhibition of NO synthesis while the second group was taken as control. They observed that after effective long-term (4 weeks) blockade of NO synthesis wall shear stress in the flow-loaded artery varied directly with blood flow; suggesting that elimination of the NO pathway significantly prevents vascular adaptation to increased blood flow.
Alterations of shear stress can have adverse effects on intimal thickening after vessel injury. Damage to the vascular endothelium triggers a complex series of events that results in abnormal migration and proliferation of smooth muscle cells with associated deposition of extracellular matrix [44]. Kohler and Jawien [45] studied the effects of blood flow on intimal hyperplasia following balloon catheter injury in rat common carotid arteries. Flow was increased by ligation of the opposite common carotid artery or decreased by ligation of the ipsilateral internal carotid artery. They observed that though neointimal hyperplasia developed in arteries exposed to increased as well as to decreased flow, early neointimal thickening was significantly enhanced in arteries exposed to low flow (fig. 10).

Figure 10: Intimal hyperplasia following balloon injury in rat carotid. The left panel represents the experimental model (X indicates site of ligation, * indicates balloon injured region). The right panel represents histological cross-sections of balloon-injured arteries submitted for 2 weeks to high and low flow respectively (IH and arrows indicate neointimal thickening in each cross-section). From [45].
Biological modulators of the arterial wall

**Matrix metalloproteinases as agents of remodeling**

All acquired evidence indicates that the major drivers of vascular remodeling, such as hemodynamic forces, injury, inflammation and oxidative stress, regulate matrix metalloproteinases (MMPs) expression and activation. Vascular remodeling entails the reconstruction of the matrix scaffolding and therefore a process of active proteolysis and resynthesis of the extracellular matrix proteins. Proteolysis of the extracellular matrix appears to be the major function exerted by MMPs, playing a main role in detachment and migration of cells, as well as tissue remodeling in several physiological and pathological situations.

**Biochemical characteristics of MMPs**

The MMPs family consists of at least 18 structurally related members. They can be defined according to several common characteristics (Table 1).

*Table 1: Common biochemical characteristics of matrix metalloproteinases*

- The catalytic mechanism depends on zinc at the active center
- The proteinases are secreted as zymogens
- The zymogens can be activated by proteinases or by organomercurials
- Activation is accompanied or followed by a loss of Mr of about 10’000
- The cDNA sequences all show homology to that of interstitial collagenase (MMP-1)
- The enzymes cleave one or more components of the extracellular matrix
• Activity is inhibited by specific tissular inhibitors (tissue inhibitors of metalloproteinases, TIMPs)

MMPs can be divided into subgroups of collagenases, gelatinases, stromelysins, and membrane-type MMPs, according to their substrate specificity and primary structure (Table 2).

Table 2: The matrix metalloproteinase family

<table>
<thead>
<tr>
<th>MMP sub-groups</th>
<th>Main name</th>
<th>Alternative names</th>
<th>MMP number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagenases</td>
<td>Collagenase-1</td>
<td>Interstitial collagenase</td>
<td>MMP-1</td>
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<td></td>
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<td>Fibroblast collagenase</td>
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<td>Vertebrate collagenase</td>
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<td>Collagenase-2</td>
<td>Neutrophil collagenase</td>
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<td>MMP-13</td>
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<td></td>
<td>Collagenase-3</td>
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<td>Gelatinases</td>
<td>Gelatinase A</td>
<td>72-kDa type IV collagenase</td>
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<tr>
<td></td>
<td>Gelatinase B</td>
<td>92-kDa type IV collagenase</td>
<td>MMP-9</td>
</tr>
<tr>
<td>Stromelysins</td>
<td>Stromelysin-1</td>
<td>Procollagen activator</td>
<td>MMP-3</td>
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<td></td>
<td></td>
<td>Proteoglycanase</td>
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<tr>
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<td>Stromelysin-3</td>
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<td>MMP-13</td>
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<td></td>
<td>Matrilysin</td>
<td>Putative metalloproteinase</td>
<td>MMP-7</td>
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<td>Uterin metalloproteinase</td>
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<td></td>
<td>Metalloelastase</td>
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<td>MMP-12</td>
</tr>
</tbody>
</table>
Membrane-type matrix metalloproteinases (MT-MMPs) | MTI-MMP | MT2-MMP | MT3-MMP | MT4-MMP
--- | --- | --- | --- | ---
 | - | - | - | -
MMP-14 | MMP-15 | MMP-16 | MMP-17

**Regulation of MMPs**

The physiological activity of MMPs must be tightly regulated in normal arteries considering that the MMP family is capable of degrading all the individual components of blood vessel extracellular matrix. An ever-increasing volume of experimental as well as clinical data illustrates the key role of MMPs in many of the processes that control vascular remodeling. The net effect of the various triggers shown to increase MMP activity is an imbalance of the ratio of MMPs to tissue inhibitors of metalloproteinases (TIMPs), in favor of extracellular matrix degradation [46].

MMPs govern processes essential for the remodeling of blood vessels. Soluble factors, cell-cell and cell-matrix interactions finely tune MMP expression and activation spatially and temporally. Although essential for the development and normal turnover of blood vessels, and beneficial for the adaptation and repair, the action of MMPs can evade normal control and thus turn the remodeling process into a pathological event. A thorough understanding of the control and consequences of MMP actions may provide new ways to manipulate vascular remodeling. New insights obtained from recent studies with MMP inhibitors and genetic manipulation suggest that depending on the setting and timing, modulation of specific MMP activities may be considered beneficial or detrimental for vascular functionality [47-50]. For example, it has been shown that MMP-9 deficiency affects arterial geometrical remodeling, mainly through modulation of collagen metabolism and organization [51]. These results suggest that specific inhibition of MMP-9 may increase the collagen content of arteries, thus potentially enhancing their mechanical stability while at the same time decreasing intimal hyperplasia and late lumen loss observed in stenotic remodeling of human arteries [51]. The difficulty in demonstrating a direct relationship between the action of MMPs and various aspects of vascular
remodeling relies on the development of specific inhibitors, appropriate animal models and diagnostic tools.

**MMPs in physiological remodeling**

MMPs play a main contribution to tissue development and tissue repair following injury [52]. The migration of arterial endothelial cells and SMC is essential in normal vessel development [53]. The formation of capillaries from preexisting blood vessels, designated as angiogenesis, is required during wound healing; it must though be emphasized that in adults vascular turnover is low and both cell types are in a quiescent state under physiological conditions [54]. The initial fragmentation of basal lamina is attributed mainly to MMPs and the main angiogenic stimuli, i.e. fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) are potent inducers of MMP expression [55]. Although MMP-1 was initially believed to be the major contributor to angiogenesis in vitro [56], other MMPs, as well as enzymes of the plasmin system, participate in angiogenesis [57] [58].

Studies done on experimental models have demonstrated that MMPs are induced in the vessel wall in response to changes in blood flow. Tronc et al. [59] investigated whether MMP activation was responsible for the adaptive vascular remodeling by treating rabbits with a compound known to have an inhibitory effect on MMP expression and activation. They observed that MMP inhibition prevented arterial wall remodeling with elastin degradation and subsequent arterial enlargement and suggested a key role for NO as a significant activator of pro-MMPs in blood flow induced vascular remodeling. Another study reported by Magid et al. [60] demonstrated that MMPs expression by endothelial cells is regulated in response to flow changes. They show that the MMP-9 gene is not only shear sensitive but that an oscillatory hemodynamic regimen exerts different effects on gene expression than does steady laminar flow.

MMPs have also been reported to be induced in the arterial wall in response to changes in pressure. Chesler et al. [61] reported that in response to experimentally induced hypertension, specific activation of MMP-2 and degradation of elastin was observed after 48 hours of in vitro culture in porcine carotid arteries.
Introduction

MMPs in pathological remodeling

Using different technical approaches, several investigators have shown that MMP expression is markedly increased in arteries of patients suffering from atherosclerosis. MMP-1, -3, and -9 levels appeared more intense in plaque shoulders and regions of foam cell accumulation [62-64]. With the exception of MMP-2, normal arteries do not express MMPs [62]; within atherosclerotic plaques, two cell types appear to produce those enzymes: SMC and macrophages. Reversal of the contractile phenotype of SMC to a motile and synthetic one is currently observed with aging and atherosclerosis. Among MMPs, gelatinases A (MMP-2) and B (MMP-9) are probably key actors in SMC migration, in keeping with their elastolytic activity, given that elastic fibers can represent the main barrier to cell locomotion [65].

Abdominal aortic aneurysm is a potentially fatal disorder characterized by a progressive degeneration of the aortic wall where destruction of medial elastin, chronic aortic wall inflammation, and the loss of smooth muscle cells is observed [66, 67]. Recent studies have emphasized the role of elastolytic proteinases in the disruption and degradation of medial elastin [68, 69]. Other studies have reported increased production of several members of the MMP family in regions of aneurismal lesions, including collagen-degrading proteinases such as collagenases and gelatinases [70, 71].

Arterial stenosis is a pathology characterized by lumen narrowing due to neointimal thickening. Histological and experimental studies have shown that proliferation and migration of vascular smooth muscle cells play key roles in neointima formation [72]. Recent works demonstrate that turnover of the extracellular basement membrane is a critical regulator of SMC migration and proliferation. In support of this concept, basement membrane-degrading metalloproteinases (MMP-2 and MMP-9) have been shown to be functionally involved in neointima formation [73, 74].

References


Functional, mechanical and geometrical adaptation of the arterial wall of a non-axisymmetric artery in vitro

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Abstract

Objective: Vascular remodeling is an adaptive response to variations in the hemodynamic environment acting on the arterial wall. Remodeling translates into changes of structure, geometry and mechanical properties of the artery. Our aim was to study the remodeling response of pig right common carotid arteries in vitro.

Methods: In vivo right carotid arteries are exposed to a non-uniform hemodynamic environment and exhibit a strong wall asymmetry in the circumferential direction that allows the study of two regions separately, as the artery remodels under in vitro perfusion. Porcine right common carotid arteries were cultured during 1(n=6), 3 (n=6) or 8 (n=6) days in an in vitro organ culture system, at a constant perfusion pressure of 100 mmHg. Geometrical, histological, biomechanical and biological analysis of the perfused segments was performed at the end of each study.

Results: Smooth muscle cell nuclei density and wall thickness remain constant along the culture periods. Elastin and collagen are significantly redistributed to equilibrate their relative content along the vessel circumference. The distensibility profile is significantly different at day 8. MMP-2 expression and activity increase significantly at day 3 and 8.

Conclusion: The non-axisymmetric arterial wall adapts to a uniform hemodynamic environment by redistributing the structural components of the extracellular matrix. The changes of collagen and elastin density may result from a vascular remodeling process involving MMP-2 up-regulation and enzymatic activity. The remodeling response results in a new vascular wall configuration more distensible at physiological pressures (30-120 mmHg) and stiffer at higher pressures.
Introduction

Arteries adapt their geometry and elastic properties when they are subjected to changes in their mechanical and hemodynamic environment such as variations in pressure and flow. Flow-induced remodeling results primarily in adaptation of vessel internal diameter aiming to normalize intimal wall shear stress [1-4]. Variations in mean blood pressure are associated to changes in wall thickness leading to normalization of wall tension [5-8]. Wall remodeling in response to acute stepwise changes in pressure or flow is a relative fast process, the bulk of the remodeling taking place within a few days following the change in the force imposed on the artery [9-11].

Studies on arterial wall remodeling have been mostly done on in vivo models, presenting the inconvenience that mechanical and hemodynamic effects cannot be separated from other systemic influences. To overcome this inherent limitation of in vivo experiments, and based on the rapidity of the remodeling response, several groups, including ours, have developed in vitro artery support systems that enable the perfusion of isolated arterial segments under precise control of the physiological, mechanical and hemodynamic environment, permitting thus to study the exact contribution of each type of mechanical force to the vascular remodeling process [12-17].

Studies on in vitro cultures have mainly focused on the functional alterations of the vascular segments exposed to changes in pressure or flow [18-24]. This is due to the fact that functional variations can be detected after few hours or days of exposure to different experimental conditions. Research on structural alterations of arteries has been harder to accomplish in vitro given the difficulties in keeping up an organ culture under optimal conditions for very long periods of time. Although remodeling responses in the cellular level can be initiated rapidly, the elicited tissue growth/degradation is often a slow process. Moreover, substantial remodeling of the vessel wall can be achieved largely through reorganization of preexisting wall elements, making the relative changes in tissue constituents small and the quantitative variations in samples cultured for few days undetectable. This is particularly true for homogeneous and axisymmetric arteries where stress and strain are uniformly distributed in the circumferential direction.
Based on the above arguments, a non-axisymmetric arterial segment can provide a useful model for monitoring rapid relative remodeling when perfused in an in vitro system, as we propose in the following study. This new model of non-axisymmetric artery considers the native circumferential asymmetry of the porcine right common carotid as an outcome of the non-homogenous mechanical and hemodynamic environment and allows the analysis of its adaptive response to an in vitro environment where mechanical and hemodynamic forces are homogenously distributed.

Materials and methods

*Model of a circumferentially asymmetric artery*

Our research is focused on the proximal segment of the porcine right common carotid that is comprised within the four centimeters following the bifurcation of the common carotid in right and left. In its native environment this segment is subjected to asymmetric conditions in shear [25, 26], to residual strain from the carotid bifurcation [27] and to asymmetric external support mainly due to the contact of the posterior area with the trachea. This unequal surrounding translates in a non-uniform vascular wall on the cross section that leads to the definition of two regions of study that for methodological purposes we have called anterior and posterior (fig. 1).

*Figure 1: Cross section of the proximal segment of the right common carotid as positioned in its native environment. The arterial wall shows a non-uniform thickness on the circumferential direction leading to the definition of two regions of study, anterior region (A) and posterior region (P). The A region is relatively thick and entirely surrounded by soft tissue while the P region is thinner, partially in contact with the trachea and closer to the bifurcation.*
Arterial preparation

Porcine carotid arteries were harvested right after sacrifice at the local slaughterhouse and rinsed with a saline solution (PBS with Ca\(^{++}\) and Mg\(^{++}\)) supplemented with 100 units/ml penicillin G sodium, 100 units/ml streptomycin sulfate, 0.75 µg/ml amphotericin B. The arteries were transported to the laboratory and kept in ice until mounting on the \textit{in vitro} perfusion system within two hours after harvesting. Before mounting the arteries, all connective tissues were removed, including most of the adventitia. A four centimeter long segment (proximal segment), starting at the bifurcation of the common carotid into right and left, was cut from the artery and mounted onto inox cannulae of 5.0 and 4.0 mm internal diameters for the proximal and distal ends of the arterial segment respectively. The cannulae were adjusted to ensure an anatomical extension factor of 1.5 [28].

Arterial \textit{in vitro} culture

Proximal segments of right common carotid arteries were cultured during periods of 1, 3 and 8 days in a pressurized \textit{in vitro} culture system developed by our group and described by Zulliger et al. [12] (fig. 2). The vessels were bathed and perfused with a nutrient rich, pH-balanced medium supplemented with 20% dialyzed new born calf serum, 100 units/ml penicillin G sodium, 100 units/ml streptomycin sulfate, 0.75 µg/ml amphotericin B. A concentration of 8% clinical grade Dextran (Sigma, Mr 65’500) was added to the medium, raising the viscosity of the nutrient liquid to 3.75 cP at 37°C, approximating typical values for human blood. The arteries cultured during 1, 3 and 8 days were submitted to 100 mmHg steady pressure. Two other groups of arteries cultured during 3 days were submitted to 30 and 170 mmHg respectively to assess the effect of pressure and \textit{in vitro} culture on the arterial response when compared to arteries cultured at 100 mmHg. Temperature (37°C), carbon dioxide concentration (5%) and shear stress (1 dyn/cm\(^2\)) were kept constant in all groups. At the end of the culture period the arterial segments were contracted with norepinephrine (3µM) to confirm smooth muscle viability and relaxed with bradykinin (4µM) to confirm endothelial viability.
Groups and sampling

The groups were formed according to the length of the culture period: 0 days, 1 day, 3 days, 8 days. The arteries cultured during 3 days were grouped according to the culture pressure: 30 mmHg, 100 mmHg and 170 mmHg. The groups of 0 days and 3 days (cultured at 100 mmHg) were composed by two subgroups of 6 right common carotids each. One group was analyzed as whole segments and in the remaining group the anterior and the posterior regions were separated to analyze them independently. The groups of 1 day, 3 days (cultured at 30 and 170 mmHg) and 8 days were composed by 6 right common carotids each that were analyzed as whole segments. The whole segments where used for histology and protein analyses and the samples were the A and P regions were separated were used only for protein analyses.
Arterial wall geometry and distensibility

Arteries were left 24 hours under the perfusion pressure and flow to stabilize and recover from harvesting, transport, and preparation. The geometry measurements were done on day 1 and on the last day of the culture period. The arterial segments were preconditioned by five pressure cycles in which the internal arterial pressure was slowly changed from 0 to 200 mmHg and back each time. The arteries were kept at 100 mmHg after the preconditioning during 15 minutes to stabilize. Then the pressure was again set to zero and the arterial wall thickness and diameter were recorded while the inflation pressure was gradually raised to 200 mmHg within 3 to 4 minutes. The internal diameter and wall thickness were assessed by a high-resolution ultrasonic echo-tracking system (NIUS 02, Omega Electronics SA ± 2 µm accuracy). After finishing the measurements, pressure was reset to the operational level. Distensibility was assessed as described in Zulliger et al. [29]

Histology

At the end of the perfusion period, a part of each right common carotid sample (whole segments) was treated for histology. Half of the arterial sample (approximately 1 cm length) was fixed in 4% paraformaldehyde, paraffin embedded and cross-sectioned at 4µm thickness (Leica Microtom RM2135). Miller’s elastic stain was used to visualize elastin [30], picro-sirius red for collagen [31] and hematoxylin-eosin for smooth muscle cell (SMC) nuclei.

Histomorphometry and statistical analysis

SMC nuclei density and elastin and collagen area measurements were done using the KS400 Image Processing and Image Analysis system (Carl Zeiss SA, Feldbach, Switzerland). The analysis of structural indicators was performed measuring ten light microscopy fields on each of the anterior (A) and posterior (P) region. One field area was of 768 µm² and ten fields together represented approximately 5% of the total arterial cross-section area. The quantities measured were analyzed in terms of ratios of the A over the P region (A/P) and were taken as estimations of the component distribution in the arterial wall. The values for each measured field were transformed using \( x' = \sqrt[2]{x} \), where \( x \) was de density of the nuclei or the percentage of collagen or elastin measured.
Mean values were calculated for the anterior and posterior regions of each sample to form the A/P ratios.

**Antibodies and chemicals**

The following products were used: purified human MMP-2 (Gelatinase A), purified human MMP-9, mouse monoclonal antibody against human MMP-2 and mouse monoclonal antibody against human MMP-9 from Chemicon International Inc (Temecula, California, USA); monoclonal antibody against β-actin from Juro Supply (Lucerne, Switzerland); mouse IgG HRP-linked antibody from Amersham (Dübendorf, Switzerland). All the chemical products for protein extraction, electrophoresis, zymography and immunoblotting were from Sigma-Aldrich (Buchs SG, Switzerland).

**Immunohistochemistry**

Immunostaining for MMP-2 was performed on paraffin sections using the Innogenex IHC kit (Innogenex, San Ramon, California, USA), following the manufacturer’s recommended procedure. Specimens were incubated for 60 minutes at room temperature with the primary antibody against human MMP-2.

**Protein extractions**

At the end of the perfusion period, the arterial samples were deep frozen in liquid nitrogen and subsequently homogenized with a Polytron homogeneiser from Polylab (Geneva, Switzerland) in a solution containing 50 mmol/L Tris pH 7.5, 1 mol/L NaCl, 2 mol/L urea, 0.1% Brij-35 and complete, mini, EDTA-free protease inhibitor cocktail from Roche (Penzberg, Germany). Unbroken cells and non-soluble extracellular matrix components were removed by centrifugation at 14’000 g, for 15 minutes at 4°C. Protein concentration in supernatants was measured with the method of Bradford, with bovine serum albumin as the standard. All tissue homogenates were stored at –80°C until use.

**Electrophoresis**

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a discontinuous system with a Mini-Protean II apparatus from Bio-Rad (Basel, Switzerland). The protein samples were normalized to total protein (10µg per lane). Purified human MMP-2 and
MMP-9 were used as positive controls (0.1 ng and 0.5 ng per lane for zymography and immunoblot respectively).

**Zymography**

To investigate MMP-2 and MMP-9 activation, proteins were separated under non-reducing conditions on 7% polyacrylamide gels that contained 0.1% (weight / volume) gelatin. After the migration the proteins were refolded in 2.5% Triton X-100 to remove SDS, two times 20 minutes, and incubated in 50 mmol/L Tris-Hcl pH 7.6, 20 mmol/L NaCl, 5 mmol/L Ca Cl₂ and 0.02% (v/v) Brij-35 overnight at 37°C to allow substrate cleavage with proteinases. The gels then were stained with 0.1% Coomassie Brilliant blue R-250 and destained in 10% acetic acid and 30% methanol in H₂O. Gelatinolytic activity was detected as unstained bands on a blue background.

**Immunoblot analysis**

For the characterization of MMP-2 the proteins were separated under reducing conditions on 10% polyacrylamide gels and electroblotted on hybond ECL nitrocellulose membranes from Amersham Pharmacia Biotech (Buckinghamshire, England), blocked with 1% Top-Block from Juro Supply (Lucerne, Switzerland) in 0.1% Tween-20, 100 mmol/L NaCl , 10 mmol/L Tris-HCl, pH 7.5 and incubated overnight at 4°C with monoclonal antibodies against MMP-2 (1:500) and β -actin (1:5000). Mouse IgG HRP-linked was used as the secondary antibody (1:5000). Immune complexes were visualized by means of enhanced chemiluminescence (ECL+ plus) kit reagents from Amersham Biosciences (Dübendorf, Switzerland).

**Densitometry**

All blots were processed with a Kodak Image Station 2000R and quantified with Kodak 1D Image Analysis Software from Kodak Scientific Imaging Systems. MMP-2 activity as visualized on gelatin zymography was calculated as the ratio of the active form over the total MMP-2 (latent + active form) on each sample. On immunoblots, expression of total MMP-2 was calculated as the sum of the ratios of latent and active forms of MMP-2 over β-actin for each sample. The calculated ratios were normalized to the average of 0 day values.
Statistics

The groups were compared using the one-way analysis of variance (ANOVA) and the unpaired student $t$-test. All values were averaged and expressed as mean plus or minus standard deviations with the level of significance set at 0.05.

Results

Quantitative analysis of the geometrical, histological and mechanical response to in vitro culture

The proximal segment of the porcine right common carotid is comprised within the four centimeters following the bifurcation of the common carotid in right and left. In vivo this arterial segment is exposed to a non-uniform hemodynamic environment and exhibits a strong wall asymmetry in the circumferential direction that allows the study of two regions, separately, as it remodels under in vitro perfusion.

The evolution of wall thickness over the cultured periods (day 1, day 3 and day 8), as an estimation of geometrical adaptation, is shown in fig. 3. Differences in A/P ratios within the groups are not significant (p-values > 0.05). For all three groups the A/P ratio values are above 1 indicating that the anterior region remains thicker than the posterior region.
Figure 3: Geometrical changes of the arterial wall over time measured as A/P ratio of wall thickness and as absolute values (mm) on A and P regions day 1, day 3 and day 8. The anterior region remains thicker than the posterior region (A/P ratio values > 1). Changes are non significant (p-values > 0.05). Bars represent averages ± SD.

In the absence of an inflammatory process, smooth muscle cells (SMC) are the main cellular component of the media of the arterial wall and together with the scleroproteins elastin and collagen constitute key elements in the tissue organization of the arterial wall. The evolution of SMC distribution over time was evaluated by measuring SMC nuclei density on the A and P regions in all three groups. Changes in A/P ratios showed to be non significant (p-values > 0.05). SMC are evenly distributed around the arterial wall from day 1 to day 8, reflected by A/P ratio values approaching 1 (fig. 4). The distribution of elastin and collagen was assessed measuring the wall area occupied by these elements on the A and P region and calculating the A/P ratio. On day 1
elastin was more abundant on the posterior region (A/P ratio value equal to 0.94± 0.11). At day 8 the A/P elastin ratio was 1.03± 0.16 suggesting that a redistribution process of elastin has taken place in response to the new hemodynamic environment, the difference being statistically significant when compared to day 1 (p-value < 0.02). Collagen exhibits a change opposite to the one of elastin, at day 1 the anterior region is richer in collagen than the posterior region (A/P ratio value equal to 1.38± 0.41). At day 8 A/P collagen ratio becomes 1.09± 0.12, suggesting that the matrix redistribution also involves collagen molecules. The difference in A/P ratio of collagen area between day 1 and day 8 is statistically significant (p-value < 0.05) (fig. 5). Histological staining for elastin and collagen at day 1 and day 8 illustrate the tendencies quantified through histomorphometry (fig. 6).

![Graph of A/P ratio of SMC nuclei density](image)

*Figure 4: Evolution of SMC distribution evaluated as the A/P ratio of SMC nuclei density at day 1, day 3 and day 8. SMCs are evenly distributed around the arterial wall along the culture periods (A/P ratio values ≤ 1, p-values > 0.05).*
Figure 5: Evolution of elastin and collagen distribution over the culture periods, measured as the A/P ratio of the areas covered by these scleroproteins. The distribution of elastin and collagen changes significantly from day 1 to day 8 (p-values < 0.02 and 0.05 respectively). Both elements tend to equilibrate on the A and P regions.

To investigate a possible biomechanical link between structural changes and distensibility, at the end of each culture period we subjected the porcine right carotid arteries to in vitro pressure inflation (0 to 200 mmHg). From the pressure-diameter curves we calculated the distensibility. The arterial segments showed an increase in diameter at day 8 and when compared to day 1. Arterial response to pressure inflation was similar at day 1 and day 3, reaching the maximum distensibility at the operating pressure (100 mmHg) (fig. 7). At day 8 the distensibility profile is significantly different from the one observed at day 1 and day 3, the maximum distensibility is shifted to lower pressures (70 mmHg) and its value is increased by 40% with respect to day 1.
Figure 6: The photomicrographs illustrate the elastin and collagen arrangements on the media of right common carotids, in the A and P regions at day 1 and day 8. The cross sections were stained with Miller’s elastic stain to evidence elastin fibers and prico-sirius red stain to reveal collagen fibers. All images were taken at x20 original magnification and were scaled identically.
Figure 7: Distensibility calculated from pressure-diameter curves traced at the end of each culture period (day 1, day 3 and day 8), diameter curve in relation to pressure at day 1 and day 8 of culture period. The distensibility profile is very similar for arteries cultured for 1 and 3 days, the maximum distensibility is reached at 100 mmHg. At day 8 the arterial response is significantly different, the distensibility peak is attained at 70 mmHg and the maximum of distensibility is increased by 40%. The diameter curve shows a significant increase in diameter at day 8. Bars represent averages ± SD.
Role of MMP-2 in the remodeling response

Controlled proteolysis of the extracellular matrix (ECM) by matrix metalloproteinases (MMPs) plays a main role in tissue remodeling. Among members of the MMP family, gelatinases A (MMP-2) and B (MMP-9) are known to degrade short collagen and elastin fibers, major components of the arterial wall in response to hemodynamic factors, such as flow or pressure [23, 32].

To determine a connection between the adaptive response of the vessel and the enzymatic effects, we assessed the activity and expression of MMP-2 and 9 on arterial segments just excised from their native environment (0 days) and arterial segments cultured during 1, 3 and 8 days. The MMP-2 gelatinolytic activity increases significantly at day 3 and persists at day 8 (fig. 8), and is coupled to a significant increase in the induction of MMP-2 at day 3 and day 8 (fig. 9). The gelatinolytic activity of MMP-9 zymogen showed to increase at day 8 (fig. 8) but could not be quantified given that the signal was very weak or absent day 0, 1 and 3.
Figure 8: MMP-2 activation rate quantified from zymography, calculated as the ratio of active over total MMP-2. The gelatinolytic activity increases significantly at day 3 and day 8. Bars represent averages ± SD. The photographic images illustrate raw SDS-PAGE zymography data showing the zymogen form (72 kDa) and the active form (62 kDa) of MMP-2 and the zymogen (92 kDa) form of MMP-9 at the different culture periods.
Figure 9: MMP-2 induction fold quantified from SDS-PAGE immunoblot, calculated as the total MMP-2 expression normalized by the average of total MMP-2 at 0 days. Normalization of protein concentration was done calculating the ratio of MMP-2 over β-actin. MMP-2 is significantly induced at day 3 and day 8. Bars represent averages ± SD. The photographic image illustrates raw SDS-PAGE immunoblot data showing the zymogen form (72 kDa) and the active form (62 kDa) of MMP-2 at the different culture periods.

Our main hypothesis for the in vitro remodeling of the non-axisymmetric carotid artery model is that the arterial segment is exposed to a totally different stress and strain field in vitro as opposed to in vivo. As shown in the literature, changes in stress and strain lead to activation of MMPs. To further support these findings in the context of our non-axisymmetric arterial segments, we performed an experiment were arterial segments were cultured during three days at different pressures, namely at 30, 100 and 170 mmHg, under exactly the same conditions than the other groups, and determined the gelatinolytic activity and expression of MMP-2. The activity of MMP-2 showed to increase as culture pressure
decreased. The increase in activation at 30 mmHg was not significantly higher when compared to the activity at 100 mmHg, but was statistically significant when compared to the activation rate at 170 mmHg (p-value < 0.01). The difference in the activation rate was also statistically significant between 100 mmHg and 170 mmHg (p-value < 0.01). MMP-2 expression did not show significant differences between the three groups (fig. 10). This result confirms that MMP-2 activation relates to a change in the biomechanical forces imposed on the arterial wall.

Figure 10: Effects of strain and stress changes on MMP-2 activation and expression, as quantified on arteries cultured during 3 days at 30, 100 and 170 mmHg. MMP-2 activation rate quantified from SDS-PAGE zymography, calculated as the ratio of active over total MMP-2, sum of the zymogen (pro-MMP2) and the active form of the enzyme. The gelatinolytic activity is higher at 30 mmHg when compared with values at 100 mmHg (non significant) and values at 170 mmHg (p-value < 0.01). MMP-2 activity is significantly higher at 100 mmHg when compared to 170 mmHg (p-value < 0.01). MMP-2 induction fold quantified from SDS-PAGE immunoblot, calculated as the
total MMP-2 expression normalized by the average of total MMP-2 at 100 mmHg. Normalization of protein concentration was done calculating the ratio of MMP-2 over β-actin. Differences between groups are non significant. Bars represent averages ± SD.

To evaluate a possible mechanism underlying the elastin and collagen redistribution observed during the structural analysis, we quantified the activity and expression of MMP-2 on the A and P walls of arterial segments just excised from their native environment (0 days) and arterial segments cultured during 1, 3 and 8 days at 100 mmHg. The results show that MMP-2 activity (fig. 11) and induction (fig. 12) increase significantly at 3 days (p-value < 0.01) on both regions A and P when compared to activity and expression levels on day 0. However, non-statistical differences were found between the A and P regions at day 0 or after 3 days of in vitro culture. Immunostaining for MMP-2 on A and P region at day 3 confirm the homogenous distribution of MMP-2 throughout the media (fig. 13).

![MMP-2 activation rate](image)

**Figure 11:** MMP-2 activation rate quantified from SDS-PAGE zymography, calculated as the ratio of active over total MMP-2. The gelatinolytic activity is significantly higher at 3 days (p-value < 0.01) in both A and P regions. Bars represent averages ± SD.
Figure 12: MMP-2 induction fold quantified from SDS-PAGE immunoblot, calculated as the total MMP-2 expression normalized by the average of total MMP-2 at 0 days. Normalization of protein concentration was calculated as the ratio of MMP-2 over β-actin. MMP-2 expression is significantly higher at 3 days (p-value < 0.01) on both regions A and P. Bars represent averages ± SD.

Figure 13: The photomicrographs illustrate the MMP-2 distribution on the media of right common carotids, in the A and P regions at day 3. The red-brown staining on the cross sections represents the MMP-2 immunopositive response; the letter L indicates arterial lumen. All images were taken at x20 original magnification and were scaled identically.
Discussion

Arterial wall remodeling is an adaptive response to changes in the mechanical and hemodynamic environment that can be characterized through reorganization of tissue structure, variations in tissue geometry and volume and mechanical changes in the properties of the vessel [9, 10]. We examined the short-term adaptation of the proximal segment of the right common carotid after in vitro perfusion during 1, 3 or 8 days. In vivo, this elastic artery is subjected to asymmetric conditions in shear [25, 26] and to asymmetric external tissue support. The structural asymmetry of the artery allows the definition of two regions, the anterior (A) and the posterior (P) (fig. 1A), which differ in their spatial surrounding, thickness and scleroprotein content. The posterior region is partially in contact with the rigid cartilaginous tissue of the trachea and in relative terms is thin, rich in elastin and poor in collagen. The anterior region is entirely surrounded by soft tissue and is relatively thick, poor in elastin and rich in collagen.

In our in vitro model we observe an adaptive response of the vascular wall after 8 days of exposure of the artery to constant pressure and shear stress. The distribution of scleroproteins (elastin and collagen) tends to equilibrate between the anterior and the posterior regions of the wall. This equilibration, however, is not accompanied by a normalization in wall thickness and smooth muscle cell distribution. The arterial distensibility changes significantly with time, the distensibility peak shifts to lower pressures and the maximal value of distensibility increases. The results show that after 8 days of exposure to in vitro hemodynamics, the arterial wall adapts by first optimizing the distribution of extracellular matrix components while geometry remains unchanged, suggesting that geometrical adaptation may require a longer period. This remodeling response gives place to a new vascular wall configuration that is more distensible at physiological pressures (30-100 mmHg) and stiffer at higher pressures.

The sequence of the events observed in our study differs from the results presented by Matsumoto and Hayashi [10] who studied the in vivo mechanical and dimensional adaptation of the rat aorta to hypertension. They observed that the dimensional adaptation associated to wall thickening occurs very rapidly while elastic properties of the arterial wall changed slowly. Matsumoto and Hayashi concluded
that the aortic wall responds to changes in the mechanical load by first adapting the wall thickness to keep stress in an optimal range and, second, by adapting the wall elasticity to maintain wall function (distensibility) at optimal levels. The apparent contradiction between the results presented by Matsumoto and Hayashi and ours may be attributable to differences in species, artery type, magnitude of the mechanical changes imposed on the artery, as well as to methodological issues (\textit{in vitro vs. in vivo}). In their study the artery has been exposed, \textit{in vivo} to a sudden rise in pressure and a dramatic increase in stress while in our \textit{in vitro} study the artery has been exposed to a different distribution of stress and strain when compared to its native environment. The increased in wall thickness observed in Matsumoto and Hayashi’s study can be explained by the fact that \textit{in vivo}, remodeling in response to hypertension is mediated by locally produced or activated autocrine / paracrine factors, such as growth factors or cytokines, along with circulating hormones [33]. In our \textit{in vitro} study the hormonal system is absent, what lead us to assume that the rapid adaptive response of the extracellular matrix is directly linked to the mechanical changes. The differences coming from methodological issues has been previously discussed by Matsumoto \textit{et al.} [34] in a study done on the mechanical and dimensional adaptation of the rabbit carotid artery cultured \textit{in vitro}. They pointed out that although the dimensional changes of the cultured artery were qualitatively similar to those observed \textit{in vivo}, some of them, particularly thickness, were not quantitatively the same, \textit{in vitro} changes being smaller than those expected \textit{in vivo}.

The evolution of distensibility that we observe from day 1 to day 8 can be explained through Dobrin’s study [35] who reported that in arteries elastin bears load at small distentions, while collagen, in particular medial collagen, bears load at large distentions. In our model, the initial non-homogenous distribution of elastin and collagen reflects the asymmetric distribution of strain and stress on the arterial wall. \textit{In vivo} excessive distension of the posterior region is limited by the rigid structure of the trachea that in a sense reduces the need for collagen protection, while elastin insures resiliency of the tissue by distributing stresses uniformly through the wall. The anterior region, where there is not external support to prevent large distensions, is reinforced by higher collagen content and greater wall thickness. On the distensibility curve (fig. 3) we observe that at day 1 and day 3, when the arterial
morphology is similar to in vivo, the maximum distensibility is reached at approximately the operating pressure (100 mmHg) beyond which point collagen fibers are engaged. At day 8 the maximum of distensibility is attained at lower pressures, approximately 70 mmHg, and distensibility is higher between 0 and 100 mmHg than the one measured at day 1 and 3. Wilson et al. [36] studied the relationship between distensibility and collagen and elastin metabolism in abdominal and aortic aneurysm and concluded that increase elastolysis is associated with increase wall distensibility, whereas increased collagen turn-over is associated with reduced distensibility. Taking into account these findings we can explain our results by considering that elastin A/P ratio has equilibrated, the arterial internal diameter has increased, as seen in pressure-diameter curves (fig. 3) and collagen is engaged earlier. Given that the increase in diameter is detectable from low pressures, we can assume that elastin has degraded, mostly on the posterior region, what explains the equilibration at 8 days. A decreased content in elastin translates into a diminished resistance of the wall at lower pressures and a shift of the pressure point at which collagen starts to bare load. The equilibration in collagen content can be explained by a synthesis of collagen fibers on the posterior region, what allows keeping smooth muscle cells density and thickness A/P ratio constant along the culture periods.

A major role for MMPs is to enable arterial remodeling through the decomposition of the existing ECM scaffold while a new ECM is synthesized and organized. Other studies have already described the influence of stress and strain on collagen and elastin metabolism through modulation of MMPs [23] [32] [37]. In the present study MMP-2 is up-regulated and activated after three days of arterial exposure to changes in stress and strain and MMP-9 is detectable at 8 days (fig. 4Aand 4B). The analyses done on the anterior and posterior walls of the artery show that the increase of MMP-2 induction and activity is equally significant on both regions. This results suggest that ECM turnover is taking place on both A and P regions of the artery, and that MMP-2 is the likely responsible for collagen and elastin degradation in the short-term remodeling. Little can be said about the role of MMP-9 since though detectable at 8 days it was not quantifiable. A possible explanation of the enhanced early expression and activation of MMP-2 as compared to MMP-9, is that MMP-2 is constitutively secreted by smooth muscle cells and might offer the most direct pathway for a short-term remodeling response to sudden hemodynamic changes affecting
the arterial media, while MMP-9 may need a longer signaling pathway. Another explanation can be related the magnitude of hemodynamic changes. Chesler et al. [23] studied the relationship between transmural pressure and matrix degrading activity in porcine arteries in vitro. Their results suggest that MMP-2 activation is significantly increased at physiological pressures (100 mmHg) when compared to high pressures and that MMP-9 activation increased at high pressures (200 mmHg). These observations are in accordance with our results (fig. 5) that show that MMP-2 activation is significantly higher at 30 and 100 mmHg when compared to 170 mmHg, though MMP-9 was not detectable.

This work proposes a new model of arterial wall remodeling where physical factors can be analyzed individually, and underlines the link between the structural changes, the biomechanical response and the enzymatic implication in the adaptive response tending to optimized the distribution of wall forces in a new hemodynamic environment. Further research on the pathways involved in MMPs regulation could bring more light into the mechanisms of arterial wall response leading to changes in the biomechanical properties of the artery. It has been previously proposed that mechanical stretch activates reactive oxygen species [38] [39], increases reactive oxygen species formation in smooth muscle cells and enhances oxidative stress within the vessel wall via a NAD(P)H dependent oxidase [37] and that oxidative stress may augment MMP expression and activity in vitro [40]. These studies have been achieved on isolated cells. Our model could constitute an interesting approach to confirm these findings in a more physiological environment where cells are integrated in complete tissue structure.

References


Differential in vitro adaptation of the arterial wall to steady and pulsatile pressure: analysis of geometrical, biomechanical and biological properties

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Abstract

Objective: Cyclic stretch due to the pulsatile nature of blood pressure is known to determine, among other biomechanical factors, the adaptive remodeling response of the vessel. Remodeling translates into changes of structure, geometry, mechanical and biological properties of the artery. Our aim was to investigate the arterial remodeling response to \textit{in vitro} steady and pulsatile perfusion pressures.

Methods: Porcine right common carotid arteries were perfused during 1, 3 and 8 days in an \textit{in vitro} organ culture system under controlled hemodynamic and environmental conditions. One group of arteries was exposed to a constant pressure of 100 mmHg while a second group was exposed to a pulsatile pressure varying between 75 and 125 mmHg, at a frequency of 1Hz. Structural as well as biomechanical parameters were considered at the end of each experiment.

Results: Arterial segments exposed during 8 days to steady pressure show a decrease in thickness and an increase in diameter, increased distensibility at low pressures and decreased distensibility at high pressures, increased collagen to elastin ratio and increased MMP-2 expression and activation. Arteries exposed during 8 days to pulsatile pressure show increased diameter, increased distensibility at high pressures, decreased collagen to elastin ratio, increased MMP-2 expression and decreased MMP-2 activation.

Conclusion: Arterial exposure to steady pressure, during 8 days, increases the elastolytic activity of the arterial wall leading to decreased wall stiffness, whereas 8 days of exposure to pulsatile pressure results in decreased elastolysis of the arterial wall and increased wall stiffness.
Introduction

The pulsatile nature of blood pressure subjects the arterial wall to cyclic stretch. Changes in the amplitude of cyclic stretch determines, among other biomechanical factors, the adaptive remodeling response of the vessel, which is characterized by modified morphology and function. During the cardiac cycle, large arteries can stretch up to 9-10%, which can be increased to 15% with hypertension [1, 2]. In vitro studies show that cyclic stretch can influence the differentiation of smooth muscle cells (SMC). In rabbit aortic SMC, cyclic stretch induces a selective and reversible accumulation of caldesmon heavy chain [3]. In rat SMC, cyclic stretch alters myosin isoform expression towards a more differentiated state [4]. Cyclic stretch has also been demonstrated to stimulate elastin and collagen synthesis in rat aortic SMC [5], nevertheless this effect was shown to depend on the matrix on which SMC were cultured.

Boutouyrie et al. [6] performed a study on elastic and muscular arteries in normotensive and never-treated hypertensive patients to determine the respective influences of local pulsatile and steady pressures on large artery remodeling. It was found that local pulsatile pressure is a strong independent determinant of common carotid artery enlargement and wall thickening. This adaptive process necessitates the breakdown and reorganization of the extracellular matrix scaffold. Increasing evidence implicates the contribution of matrix metalloproteinases (MMPs) in the wall remodeling in response to cyclic stretch [7]. In vitro studies on SMC isolated from human saphenous veins have shown that cyclic strain down regulates the production of MMPs by decreasing MMP-2 expression as opposed to stationary strain that increases MMP-2 expression and activation [8]. Other studies done on mice aortic SMC have demonstrated that mechanical stretch elicits MMPs expression and activation via NAD(P)H oxidase-derived reactive oxygen species [9].

There is growing indirect evidence leading to the conclusion that cyclic stretch could be a major determinant of arterial remodeling [7, 8, 10, 11]. The suggested findings have the inherent limitations of either cellular in vitro studies where cells are isolated from their native immediate environment and deprived of the related biochemical interactions, or in vivo studies where neurohumoral and other regulatory factors added to
methodological constraints influence the remodeling response and its interpretation.

Some working groups, including ours [12-18], have developed a methodological alternative that allows to bridge the gap between isolated cells and in vivo studies. This is an in vitro organ culture system, which enables the perfusion of isolated arterial segments under precise control of the physiological, mechanical and hemodynamic environment, permitting thus to study the exact contribution of each type of mechanical force to the vascular adaptive process.

The present study aims to determine the respective influence of pulsatile and steady mechanical load on vessel wall remodeling by investigating the role of continuous and cyclic stretch on structural and geometrical adaptation, biomechanical response, extracellular matrix turnover and SMC phenotype modulation on in vitro cultured arterial segments.

**Methods**

*Arterial preparation and culture*

Proximal segments of porcine right common carotid arteries were harvested right after sacrifice at the local slaughterhouse and set in in vitro culture in a pressurized system developed by our group and described by Zulliger et al. [13]. Arteries were submitted to two different pressure treatments during 1, 3 and 8 days respectively. The first group was exposed to a steady pressure of 100 mmHg and the second group was exposed to a pulsatile pressure of 100 mmHg ± 25 mmHg, resulting in a systolic pressure of 125 mmHg and in a diastolic pressure of 75 mmHg. Pulsatility was produced and controlled by means of a pulsatile blood pump connected to the pressure system of the perfusion circuit (Harvard Apparatus Pulsatile Blood Pump). At the end of the culture period the arterial segments were contracted with norepinephrine to confirm smooth muscle viability and relaxed with bradykinin to confirm endothelial viability. Only viable arteries, in number of 6 samples for each condition, where chosen for the study. Were considered viable arteries those were culture was absolutely exempt of signs of contamination and were the tissue evidenced SMC and endothelium functionality through drug tests. The groups were formed according to
the length of the culture period: 1 day, 3 days, 8 days and to pressure treatment: steady and pulsatile pressure. A group of freshly harvested arterial segments (0 days) was included as control in the biochemical analysis of the samples.

**Histology and Histomorphometry**

At the end of the perfusion period, a part of each right common carotid sample was treated for histology as described by Zulliger et al. [13]. Miller’s elastic stain was used to visualize elastin [19] and picro-sirius red for collagen [20]. Elastin and collagen area measurements were done using the KS400 Image Processing and Image Analysis system (Carl Zeiss SA, Feldbach, Switzerland). The quantitative analysis was performed measuring twenty light microscopy fields homogenously distributed throughout the arterial cross section. One field area was of 768 µm² and twenty fields together represented approximately 10% of the total arterial cross section area. The values for each measured field were transformed using \( x' = \sqrt{x} \), where \( x \) was the percentage of collagen or elastin measured. Mean values were calculated for the total arterial cross section.

**Geometrical and mechanical properties of the arterial wall**

Wall geometry and distensibility were assessed as described in Zulliger et al. [21]. The modulus of elasticity (\( E_{\text{inc}} \)) was calculated using \( \frac{3}{2} \left( \frac{r_i^2}{r_o} \right) \frac{\Delta P}{(r_o^2 - r_i^2) \Delta r_o} \) where \( r_i \) is the internal arterial radius, \( r_o \) is the external arterial radius and \( P \) is pressure. The modulus of elasticity was traced as a function of the circumferential stretch ratio (\( \lambda_{\theta} \)) which was calculated as the ratio of the mean arterial radius to the mean arterial radius at 0 mmHg. The mean arterial radius was calculated using \( r_i + h/2 \) where \( r_i \) is the internal arterial radius and \( h \) is the arterial wall thickness.

**Protein extractions**

At the end of the perfusion period, the arterial samples were deep frozen in liquid nitrogen and subsequently homogenized with a Polytron homogeneiser from Polylabo (Geneva, Switzerland) in a solution containing 50mmol/L Tris pH7.5, 1mol/L NaCl, 2mol/L urea, 0.1% Brij-35 and complete, mini, EDTA-free protease inhibitor cocktail from Roche (Penzberg, Germany). Unbroken cells and non-soluble extracellular
matrix components were removed by centrifugation at 14’000 g, for 15 minutes at 4°C. Protein concentration in supernatants was measured with the method of Bradford, with bovine serum albumin as the standard. All tissue homogenates were stored at –80°C until use.

Zymography

Gelatinolytic activity of MMP-2 was analyzed through zymography as described by Annabi et al. [22].

Immunoblot analysis

For the characterization of MMP-2 and smoothelin, the proteins were separated under reducing conditions on 10% polyacrylamide gels and electrobotted on hybond ECL nitrocellulose membranes from Amersham Pharmacia Biotech (Buckinghamshire, England), blocked with 1% Top-Block from Juro Supply (Lucerne, Switzerland) in 0.1% Tween-20, 100 mmol/L NaCl, 10 mmol/L Tris-HCl, pH 7.5 and incubated overnight at 4°C with monoclonal antibodies against MMP-2 (1:500), smoothelin (1:200) and β -actin (1:5000). Mouse IgG HRP-linked was used as the secondary antibody (1:5000). Immune complexes were visualized by means of enhanced chemiluminescence (ECL+ plus) kit reagents from Amersham Biosciences (Dübendorf, Switzerland).

Densitometry

All blots were processed with a Kodak Image Station 2000R and quantified with Kodak 1D Image Analysis Software from Kodak Scientific Imaging Systems. MMP-2 activity as visualized on gelatin zymography was calculated as the ratio of the active form over the total MMP-2 (latent + active form) on each sample. On immunobLOTS, expression was calculated as the ratio of total MMP-2 over β-actin for each sample. On smoothelin immunobLOTS only smoothelin-B (100 kDa) induction was measured calculating the ratio of smoothelin-B expression over β-actin. The calculated ratios were normalized to the average of 0 day values.

Statistics

The groups were compared using the one-way analysis of variance (ANOVA) and the unpaired student t-test. All values were averaged and
expressed as mean plus or minus standard deviations with the level of significance set at 0.05.

Results

Geometrical adaptation

Geometrical changes in response to pressure treatment were characterized by assessing mean arterial diameter and wall thickness. Pressure-diameter curves measured for the steady and pulsatile pressure groups between 70 and 150 mmHg are shown in figure 1. In the steady pressure group the diameter measured at day 8 appears to increase for all pressure points when compared to day 1 (*p< 0.05). In the pulsatile pressure group the diameter measured at day 8 shows a significant increase at low pressures (P< 90 mmHg) (*p< 0.05) and no significant differences between at higher pressures (P> 90 mmHg).

Pressure-thickness curves measured between 70 and 150 mmHg for both steady and pulsatile pressure groups are shown in figure 2. Thickness measured after 8 days of steady pressure treatment shows a significant decrease for all pressure points when compared to day 1 (*p< 0.05). Thickness measured after 8 days of pulsatile pressure treatment shows no significant difference when compared to day 1. Absence of vasodilatory effect of perfused medium (data not shown) supports the notion that changes in diameter during time are only determined by mechanical load.
Figure 1: Evolution of diameter in arterial segments cultured in vitro during 1 and 8 days under steady or pulsatile pressure. In the steady pressure group the diameter measured at day 8 increases for all pressure points when compared to day 1 (* p< 0.05). In the pulsatile pressure group the diameter measured at day 8 shows a significant increase at low pressures (P< 90 mmHg) (* p< 0.05) and no significant differences at high pressures (P> 90 mmHg) when compared to day 1. Curves represent averages ± SD.
Figure 2: Evolution of wall thickness in arterial segments cultured in vitro during 1 and 8 days under steady or pulsatile pressure. Thickness measured after 8 days of steady pressure treatment shows a significant decrease for all pressure points when compared to day 1 (*p < 0.05). Thickness measured after 8 days of pulsatile pressure treatment shows no significant difference when compared to day 1. Curves represent averages ± SD.
Biomechanical properties

To characterize the adaptive remodeling of the arterial wall in response to steady and pulsatile pressure from a biomechanical perspective, we assessed the distensibility and the incremental elastic modulus of the arterial wall. The distensibility-pressure curves for pressures ranging between 70 and 150 mmHg are shown in figure 3 for both steady and pulsatile perfusion pressure groups. Under steady perfusion pressure, the distensibility-pressure curve changes dramatically at day 8, being the distensibility significantly higher below 110 mmHg and significantly lower above 140 mmHg when compared to day 1 (*p< 0.05). In the pulsatile pressure group, the distensibility decreases for all pressures at day 8, being the decrease more significant as pressure increases and above 90 mmHg (* p< 0.05).

The incremental elastic modulus calculated as a function of the circumferential stretch ratio ($\lambda_{\theta}$) for steady and pulsatile pressure groups is shown in figure 4. Arteries exposed during 8 days to steady pressure show a decrease in the incremental elastic modulus as compared to day 1. The incremental elastic modulus calculated for arteries exposed during 1 and 8 days to pulsatile pressure is identical in both groups at low stretch ratios ($\lambda_{\theta} < 1.3$) but increases in the 8 day group at high stretch ratios ($\lambda_{\theta} > 1.3$). The changes observed in the evolution of the elastic modulus in both steady and pulsatile pressure groups are not statistically significant.
Figure 3: Evolution of the biomechanical behavior in response to steady and pulsatile pressure treatment. Distensibility calculated from pressure (70 to 150 mmHg) - diameter curves traced at the end of the culture period (day 1 and day 8). In the steady pressure group the distensibility profile changes dramatically at day 8 as compared to day 1, being the values significantly higher below 120 mmHg and significantly lower above 140 mmHg (* p < 0.05). In the pulsatile pressure group the distensibility decreases significantly at day 8 when compared to day 1, being the difference significant as pressure increases (* p < 0.05). Curves represent averages ± SD.
Figure 4: Evolution of the biomechanical behavior in response to steady and pulsatile pressure treatment. The incremental elastic modulus ($E_{inc}$) is traced as a function of the circumferential stretch ratio ($\lambda_\theta$) for steady and pulsatile pressure groups. Arteries exposed during 8 days to steady pressure show a decrease in the incremental elastic modulus as compared to day 1 for the entire range of circumferential stretch. The modulus of elasticity calculated for arteries exposed during 1 and 8 days to pulsatile pressure is identical in both groups at low circumferential stretch values ($\lambda_\theta < 1.3$) but increases in the 8 days group as the stretch increases ($\lambda_\theta > 1.3$). The changes observed in the evolution of the elastic modulus in both steady and pulsatile pressure groups are not statistically significant. Curves represent averages ± SD.
Evolution of relative content of structural proteins

Scleroproteins collagen and elastin are the main structural proteins of the porcine carotid media. A semi-quantitative analysis of total collagen to elastin area ratio suggests differential responses to steady and pulsatile pressure. In arteries exposed to steady pressure the total collagen to elastin ratio increases significantly from day 3 to day 8 (p< 0.025) of in vitro culture, though the difference is not significant between day 1 and day 8. In arteries exposed to pulsatile pressure the total collagen to elastin ratio decreases significantly at day 8 of in vitro culture when compared to day 1 and day 3 (p< 0.001) (fig. 5).

MMP-2 induction and activation in response to steady and pulsatile pressure

MMP-2 is a matrix metalloproteinase constitutively expressed in the arterial media and is known to play a key role in vascular remodeling [9, 23]. To investigate mechanisms underlying extracellular matrix turnover in response to steady and pulsatile pressure, we assessed the expression and activation of MMP-2 on arterial segments just excised from their native environment (day 0) and arterial segments cultured during 1, 3 and 8 days under steady and pulsatile pressure. MMP-2 is significantly induced after 3 days of in vitro culture in arteries exposed to both steady (p< 0.005) and pulsatile pressure (p< 0.001). This significant increase in MMP-2 induction is further augmented after 8 days of culture in both groups (fig. 6).

The MMP-2 gelatinolytic activity increases significantly after 3 days of culture in both steady and pulsatile pressure groups (p< 0.001). The increased MMP-2 activation is maintained during 8 days of culture in the steady pressure group but a significant decrease from day 3 to day 8 is observed in the pulsatile pressure group (p< 0.04) (fig. 7).
Figure 5: Evolution of relative content of structural proteins in response to steady and pulsatile pressure treatment. In arteries exposed to steady pressure the ratio of total collagen to elastin area increases significantly from day 3 to day 8 ($p < 0.025$) of in vitro culture, though the difference is not significant between day 1 and day 8. In arteries exposed to pulsatile pressure the ratio of total collagen to elastin area decreases significantly at day 8 of in vitro culture when compared to day 1 and day 3 ($p < 0.001$). Bars represent averages ± SD.
Figure 6: MMP-2 expression as quantified on arteries immediately following harvesting (day 0) and after 1, 3 and 8 days of in vitro exposure to steady or pulsatile pressure. MMP-2 induction fold was quantified from SDS-PAGE immunoblot, calculated as the total MMP-2 normalized by the average of total MMP-2 at 0 days. Normalization of protein concentration was done calculating the ratio of MMP-2 over β-actin. MMP-2 is significantly induced after 3 days of culture and increases further at day 8 in both steady and pulsatile pressure groups. Bars represent averages ± SD.
Figure 7: MMP-2 gelatinolytic activity as quantified on arteries immediately following harvesting (day 0) and after 1, 3 and 8 days of in vitro exposure to steady or pulsatile pressure. MMP-2 activation rate was quantified from zymography, calculated as the ratio of active over total MMP-2. In the steady pressure group MMP-2 is significantly activated at day 3 and day 8 (p< 0.001). In the pulsatile pressure group MMP-2 activity increases significantly at day 3 when compared to day 0 and day 1 (p< 0.001) but shows a significant decrease at day 8 (p≤ 0.04) when compared to day 3. Bars represent averages ± SD.
Modulation of SMC phenotype in response to steady and pulsatile pressure

The exertion of a periodic dynamic strain on the arterial wall is hypothesized to be relevant to SMC morphology and function [24-26]. We analyzed the expression of smoothelin, a proven marker of SMC differentiation [27]. To investigate the influence of pulsatile pressure on SMC phenotype modulation, we assessed the expression of smoothelin B on arterial segments just excised from their native environment (day 0) and arterial segments cultured during 8 days under steady and pulsatile pressure. Smoothelin B expression appears to be constant from day 0 to day 8 in both steady and pulsatile pressure groups, suggesting that pulsatile pressure does not have a regulatory effect on SMC differentiation (data not shown).

Discussion

In the present work we investigated the effects of steady and pulsatile pressure on vessel wall remodeling by analyzing the role of continuous and cyclic stretch on arterial structural and geometrical adaptation, biomechanical response, extracellular matrix turnover and SMC phenotype modulation on in vitro cultured arterial segments.

Cyclic stretch due to pulsating pressure is a determinant biomechanical stimulus acting on wall function and on the vascular remodeling response. There is considerable evidence that cyclic stretch can substantially alter the growth and phenotype of isolated SMC in culture [3, 4] and influence extracellular matrix turnover [7-9]. Moreover, in vivo studies have shown that cyclic stretch due to pulsatile pressure affects arterial geometry [6]. In the present study we have characterized the arterial adaptive response to steady and pulsatile pressure by interrelating the geometrical and structural changes with the biomechanical behavior. We have as well evaluated the implication of MMP-2 in the biological mechanisms underlying the observed remodeling response.

Biomechanical adaptation to steady pressure

Arterial segments exposed to steady pressure for 8 days show an increase in diameter and a decrease in thickness, the differences being
statistically significant for all pressure points when compared to the corresponding diameter and thickness values at day 1 (fig. 1 and 2). After 8 days of exposure to steady pressure the distensibility profile is very different as compared to the distensibility profile at day 1 (fig. 3). Distensibility has increased significantly at low pressures ($P<110$ mmHg) and decreased significantly at high pressures ($P>140$ mmHg). The changes in thickness, diameter and distensibility in arteries exposed to steady pressure are reflected in the evolution of the modulus of elasticity (fig. 4). Though the differences are not significant, after 8 days of exposure to steady pressure, the elastic modulus has consistently decreased as compared to day 1 for the entire range of circumferential stretch. This means that exposure to steady perfusion pressure in vitro renders the wall material more supple.

The observed biomechanical changes in terms of elastic modulus and distensibility can be related to the structural evolution of the arterial wall. As shown in figure 5, after 8 days of exposure to steady pressure the relative elastin content has decreased and the relative collagen content has increased as compared to day 1 where both scleroproteins were present in similar amounts. It may seem paradoxical that a relative increase in collagen leads to a suppler arterial wall. However, close inspection of the pressure-diameter curve (fig. 1) as well as the incremental elastic modulus ($E_{inc}$) -circumferential stretch ($\lambda_0$) curve (fig. 4) reveals that the structural response and the material properties of pig carotid arteries are dominated by elastin. This is evidenced by the fairly linear pressure-diameter curve and the relative flat $E_{inc} - \lambda_0$ curve, which suggests that for the physiological pressure and deformation levels studied here collagen fibers did not engage in bearing load, in which case we would have observed a diameter limitation at high pressures as well as an abrupt increase in $E_{inc}$ at high $\lambda_0$ values. Hence, for an elastin dominated artery, a relative loss of elastin content would lead to a supple material and in consequence to a more distensible structure at the low pressure range where collagen is not yet engaged. These observations are consistent with the notion that increased elastolysis is associated with vessel dilation [28] and increased distensibility [29].

**Biomechanical adaptation to pulsatile pressure**

As for arteries perfused under steady pressure, arteries exposed during 8 days to pulsatile pressure show an increase in diameter and a
decrease in thickness when compared to day 1. The difference between day 1 and day 8, however, is not significant and tends to diminish at high pressures for both diameter and thickness (fig. 1). Arterial distensibility at day 8 is decreased, the decrease being significant at pressures higher than 90 mmHg (fig. 3). The modulus of elasticity does not change after 8 days of exposure to pulsatile pressure; however, there is a clear tendency to increase at high stretch levels ($\lambda_{ji} > 1.3$) (fig. 4).

As for the steady pressure group, the observed biomechanical changes in elastic modulus and distensibility can be related to the structural evolution of the arterial wall. In contrast to the effects of steady pressure perfusion, arterial exposure to pulsatile pressure during 8 days results in an increased relative content of elastin and a decreased relative content of collagen (fig. 5). In an elastin-dominated structure, this translates into a less distensible wall. Indeed, a higher elastin content renders the arterial wall more resistant to pressure inflation [30] and therefore less distensible. Further, the engagement of collagen at high stretch ratios increases wall stiffness and leads to a relative limitation of diameter increase at high pressures, as evidenced in the pressure diameter curve (fig. 1).

**Role of MMP-2 in arterial wall adaptation to steady and pulsatile pressure**

MMP-2 has been described as one of the major contributors to arterial extracellular matrix turnover and therefore to vascular remodeling in response to stretch [23, 31]. In our study, arteries exposed to both steady and pulsatile pressure show a time dependent increase in MMP-2 induction (fig. 6). However, MMP-2 activation increases after 3 days of exposure to both steady and pulsatile pressure, being followed by a significant decrease at 8 days in the pulsatile pressure group (fig. 7). Asanuma et al. [8] studied the effect of stationary and cyclic strain on human vascular SMC and showed that after 3 days of in vitro cell culture cyclic strain decreased the expression of MMP-2, whereas stationary strain increased the level of latent and active MMP-2, when compared with conventional static cell culture conditions. Seliktar et al. [32] studied the role of MMP-2 in the remodeling of SMC-seeded vascular constructs subjected to cyclic strain and found that 4 days exposure to cyclic strain increased both the induction and activation of
MMP-2. Our findings on the effects of steady pressure on MMP-2 induction and activation support the observations done by Asanuma et al. [8]. On the contrary, our findings on the effect of cyclic stretch on MMP-2 expression and activation differ from those reported by Asanuma et al. [8] but support the observations done by Seliktar et al. [32] probably due to the closer similarity of the chosen experimental conditions.

The changes assessed in MMP-2 induction and activation may relate to the histological changes observed in the arterial wall. Namely, in the steady pressure group, the relative loss of elastin content may be coupled to the increased activation of MMP-2. In the pulsatile pressure group the arterial wall does not show a loss of elastin. This observation may be linked to the overall reduced elastolytic activity evidenced by a significant decrease of MMP-2 activation from day 3 to day 8 of in vitro perfusion with pulsatile pressure. However, we cannot exclude the possibility that longer exposure to pulsatile pressure may induce an increase of the elastolytic activity in the arterial wall, as MMP-2 expression appears to augment in a time dependent manner.

**SMC phenotype modulation in response to steady and pulsatile pressures**

SMC phenotype modulation was examined using the contractile phenotype marker protein smoothelin B. Our results show that the induction levels of this protein do not change after 8 days of arterial exposure to in vitro steady or pulsatile perfusion pressures, suggesting that SMC phenotype is not affected by stretch treatment. Published data are contradictory in this subject. Though there is a predominating notion that periodic deformation induces SMC differentiation [4] [10], it has also been reported that cyclic stretch has no effect on some specific proteins of differentiated phenotype. Birukov et al. [3] reported that cyclic stretch regulates caldesmon heavy chain expression but has no effect on smooth muscle myosin heavy chain and on calponin regulation on rabbit vascular SMC cultured in vitro. The divergence of the results reported through literature and those of our study probably relate to methodological issues, culture of isolated SMC on synthetic matrices versus culture of entire organs, and underline the role of specific interactions between the extracellular matrix and the SMC. Extracellular matrix receptors, when coupling cells to the matrix, are plausible
transducers of stretch. These receptors, the integrins, are transmembrane proteins capable of linking the extracellular matrix to the actin cytoskeleton, thus intracellularly transmitting deformation signals of the cytoskeleton [33-35]. The links existing between the cell and the extracellular matrix remain intact in arterial segments but are broken when cells are isolated to be cultured in vitro thus altering the cell capacity to sense mechanical changes and therefore modifying the phenotype modulation response.

In conclusion, we observe that 8 days of exposure to steady pressure increases the elastolytic activity of the arterial wall leading to decreased wall stiffness, whereas 8 days of exposure to pulsatile pressure results in decreased elastolysis of the arterial wall and increased wall stiffness. MMP-2 activation is influenced by stretch treatment and appears to be related to the histological and therefore to the biomechanical changes observed, suggesting that MMP-2 dependent remodeling affects the material properties of the arterial wall. However, MMP-2 induction and SMC phenotype modulation are not influenced by steady or pulsatile pressure. Further research on the specificity of the cellular response to externally applied mechanical stress can bring new elements to the underlying mechanisms implicated in vascular remodeling. Depending on the mode of the mechanical stimulus, as well as on the cell type, a complex array of mechanotransduction pathways seems to be triggered. In vascular SMC, cytoskeletal proteins transmit and modulate the tension between focal adhesion sites, integrins and the extracellular matrix ultimately leading to adaptation of the arterial wall to the new hemodynamic conditions imposed.

References


Plaque - prone flow conditions induce endothelial dysfunction in pig carotid arteries perfused in vitro

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Abstract

Background: Hemodynamic forces play an active role in vascular pathologies, particularly in relation to the localization of atherosclerotic lesions. It has been established that a disturbed shear stress characterized by a cyclic reversal flow affects the endothelial cell and leads to a changes in arterial function and nitric oxide production.

Objective and methods: The aim of the study was to analyze endothelial and smooth muscle cell function in correlation with eNOS gene expression and arterial remodeling in arterial segments exposed to different flow patterns. To perform such analysis, arterial segments of the left common porcine carotid were mounted on an ex-vivo arterial support system (EVASS) and perfused for one and three days under unidirectional high and low shear stress (6±3 dynes/cm² and 0.3±0.1 dynes/cm²) and oscillatory shear stress (0.3±3 dynes/cm²), this latter condition mimics plaque-prone area.

Results: After three days of perfusion, endothelial function was drastically decreased in arteries exposed to oscillatory shear stress. Impaired NO-mediated vasorelaxation was correlated to lower expression of eNOS gene. SMC contractility, phenotype and proliferation were not affected by the different flow patterns. MMPs-2 and-9 expression and activation propose that this hemodynamic environment triggered a remodeling process.

Conclusion: These data suggest that oscillatory shear stress affects endothelial but not smooth muscle function along this period of perfusion and that endothelial dysfunction is directly mediated by a down-regulation of eNOS gene expression. This study further supports the hypothesis that flow plays an active role on the pathophysiology of atherosclerosis.
Introduction

Atherosclerosis is a complex multifactor disease in which the immunological and inflammatory response plays a major role. Several risk factors, including hypertension, smoking, hyperlipidemia, etc., have been associated with the initiation and progression of atherosclerotic plaques. Exposure to these risk factors is systemic, which cannot explain the focal nature of plaque development. Indeed, atherosclerotic plaques usually develop in specific areas characterized by a perturbed hemodynamic environment, such as near to branching points or areas with strong curvature. This observation strongly suggests an active role of hemodynamic forces in predisposing some areas of vascular tissue to plaque development. Furthermore, it implies the capacity of the endothelium to discern between different hemodynamic environments. Plaque-prone areas are associated with disturbed flow characterized by low mean shear stress levels and a cyclic reversal of flow direction. In contrast, plaque-free areas are associated with unidirectional flow with high shear stress, which exerts a protective effect on arterial wall.

The eNOS enzyme is the major source of nitric oxide in the vascular endothelium. Changes in its enzymatic function are likely to form the basis of alterations in NO production and, consequently endothelial function. Endothelial dysfunction with loss of endothelium-dependent vasodilatation occurs in a number of cardiovascular diseases and it is known to influence vessel wall metabolism. NO plays an essential role in mediating many effects of flow, including vasorelaxation, inhibition of apoptosis, inhibition of platelets aggregation, and anti-inflammatory action. In vitro experiments have shown that unidirectional shear stress stimulation of NO production is mediated both by an up-regulation of endothelial nitric oxide synthase (eNOS) gene expression as well as by an activation of this enzyme. On the contrary, oscillatory shear stress, mimicking plaque-prone hemodynamics, failed to induce eNOS gene expression. Several genes coding for cytoskeletal proteins (Macrophage capping protein/Cap G), for growth factors (Preproendothelin-1 gene), for proteins involved in coagulation process (Tissue factor), or for adherence proteins (VCAM-1) are also differentially regulated by unidirectional and oscillatory flows. Broader analysis of gene expression have further confirmed this selective sensitivity. Mechanical factors, such as shear stress, pressure, and cyclic stretch
also act at the level of the media, involving mainly smooth muscle cells. The changes induced on the vessel wall may be mediated in part directly by the hemodynamic environment and in part due to the changes in endothelium metabolism. Adaptations to shear stress changes, as may occur in some pathological conditions, has been shown to involve vessel wall remodeling via cell proliferation, apoptosis and matrix degradation and synthesis.

In accordance with what has been mentioned above, the present study was designed to determine that oscillatory shear stress combined with a reversal flow effectively affects endothelial function and that this action is partly mediated by a lower expression of eNOS gene, as compared to arteries exposed to unidirectional shear stress. In order to test this hypothesis, we submitted porcine arterial segments to plaque-free (unidirectional shear stress) and plaque-prone (oscillatory shear stress) conditions for one and three days using an ex vivo arterial support system (EVASS). This perfusion system has been adapted in our laboratory in order to generate a wide spectrum of mechanical forces combinations on intact arteries. By the use of such device, we were able to follow endothelial and medial adaptation to different hemodynamic environments.

Material and Methods

Arterial perfusion system

Segments of left internal porcine carotid arteries proximal to the bifurcation were obtained at the local slaughterhouse. The arteries were harvested and mounted on the perfusion system called EVASS. The EVASS system and the method have been described in detail in previous papers. The perfusion system is shown schematically in figure 1A. The arteries were perfused with M199 EBS medium containing, 5% fetal bovine serum, 10 mmol/l HEPES, 20 mg/ml gentamicin, 100 units/ml penicillin, 100 units/ml streptomycin, 0.75 µg/ml amphotericin B, and 8% medical-grade dextran (Mr 70'000, Sigma) was added to increase the medium viscosity to blood viscosity level (µ ≈ 0.04 Ns/m²). The medium was constantly gassed by 10% CO₂ / 90% air. The perfusion chamber containing the arteries was kept in an incubator at 37°C. Perfusion flow was provided by a gear pump system (Ismatec). The pump was controlled by a function generator (Hewlett Packard)
producing a 1Hz sinusoidal flow rate. Flow rate was measured with ultrasonic flow meters (Transonic System INC). Artery diameters were measured using ultrasonic echo tracking device (NIUS, Asulab). Perfusion pressure was set at 70 mmHg.

Figure 1A: Ex Vivo arterial Support System: 1) arterial segment, 2) nutrient bath and artery support, 3) perfusion liquid reservoir, 4) gas controlled input, 5) gear pump, 6) function generator, 7) air chamber, 8) mercury manometer, 9) air filter, 10) membrane pressure, 11) ultrasound probe, 12) connection to data logging computer, 13) pressure monitor, 14) dashed line indicates incubator boundary (37°C, 5% CO2, controlled humidity).

Groups and sampling

The groups were formed according to the length of the culture period: 0 days, 1 day, 3 days; and to shear stress patterns. The different levels of shear stress assessed can be classified into three categories: a unidirectional high shear stress (6± 3 dynes/cm²) with a high mean and
a high pulsatility shear stress components, a unidirectional low shear stress (0.3±0.1 dynes/cm²) with a low mean and a low pulsatility shear stress components, and oscillatory shear stress (0.3±3 dynes/cm²) with a low mean and a high pulsatility shear stress components, leading to a flow reversal over part of the pulse cycle. The three-perfusion flow patterns are shown schematically in figure 1B. Six arteries (n=6) were studied in each group.

![Diagram](image)

**Figure 1B:** Schematic representation of the three shear stress patterns studied: unidirectional high shear stress (6±3 dynes/cm²), unidirectional low shear stress (0.3±0.1 dynes/cm²), and reversal oscillatory shear stress (0.3±3 dynes/cm²).

**Endothelial and SMC function analysis**

To determine vascular contractility and endothelial NO-mediated vasorelaxation, arterial rings, of a length of 0.5 cm, were cut from the arterial segments and tested before and after each perfusion experiment. The rings were mounted in an organ chamber (Radnoti) and equilibrated in Kreb's solution (118 10⁻³ mol/L NaCl, 4.8 10⁻³ mol/L KCl, 2.5 10⁻³ mol/L CaCl₂, 1.2 10⁻³ mol/L MgSO₄, 1.2 10⁻³ mol/L KH₂PO₄, 24 10⁻³ mol/L NaHCO₃, 11 mol/L glucose, 500 1.2 10⁻³ mol/L EDTA) maintained at 37°C and constantly gassed with a 5% CO₂/ 95% O₂ mixture. Resting tension was adjusted to two grams. Arterial rings were pre-contracted in presence of 90 mmol/L KCl until a constant contraction
level was reached. A dose-response curve to norepinephrine (NE) was then determined and normalized to maximal contraction obtained with 90mM KCl. After extensive washing, arteries were precontracted with NE, in order to achieve 50-60% of maximal contraction obtained with 90 mmol/L KCl and further relaxed by increasing concentrations of bradykinin (BK). A dose-response curve to BK was then determined and normalized to the precontraction obtained with a defined dose of NE. Tension evolution was sampled at a rate of 1HZ and recorded by the use of IOX Data software, version 1.7.0 (EMKA Technology, Paris, France) and were afterward analyzed with Datanalysis 1.80.03 (EMKA Technology, Paris, France) to obtain the curve- response dose.

Immunoblotting and zymography

Protein expression and gelatinolytic activity were assessed using immunoblot and zymography techniques, respectively. Protein levels expression was quantified using a Kodak Image Station 2000R and with Kodak 1D Image Analysis Software from Kodak Scientific Imaging Systems. On immunoblots, protein expression of total eNOS was calculated as the ratio of eNOS over VE-cadherin for each sample. Protein expression of total VE-cadherin, smoothelin B, and MMPs were calculated as the ratio of the specific protein over β-actin for each sample. On zymographie, the MMPs activity was calculated as the ratio of the active form over the total MMPs (latent + active form) on each sample. The calculated ratios were normalized to the average of the control group at 0 day.

Cryofixation and Immunofluorescence

To determine the localisation of MMP-9 and the eventual proliferation of smooth muscle cells through the Ki67 marker, the immunofluorescence technique has been utilized. At the end of the perfusion period part of the arterial segments was rinsed with 0.9% NaCl, snap-frozen in OCT compound (Tissue-Tek) and stored at –80 °C until use. For Ki67 staining, serial sections of 5 µm were cut, air-dried, and fixed in acetone for 5 minutes at -20°C. Sections were incubated successively with 0.1% Triton X-100 in PBS for 10 minutes, then incubated 60 minutes with Ki67 (1:40) or MMP-9 (1:20) as primary antibody, 10% normal rabbit serum, 0.1% Triton X-100 in PBS. The sections were consecutively incubated with anti-mouse IgG rabbit fluorescing conjugate (1:100) as secondary antibody, 10% normal rabbit serum, 0.1% Triton X-100 in
PBS during 45 minutes. All steps were performed at room temperature, and between the incubation steps the cells were rinsed with PBS. Replacing the primary antibody with preimmune serum checked specificity of Ki67 or MMP-9 labelling. Sections were examined on a Zeiss Axiovert 135 microscope equipped with fluorescence and appropriate filters. Photographs were taken on a computer with use of a CCD camera (Hamamatsu) and Openlab image software (version 3.1.2).

**Antibodies and chemicals**

Mouse monoclonal anti-eNOS from BD Transduction Laboratories, mouse monoclonal anti-human MMP-2, mouse monoclonal antibody against human MMP-9, mouse monoclonal anti-smoothelin B, and mouse anti-Ki 67 were obtained from Chemicon International; mouse monoclonal anti-β-actin from Cytoskeleton; mouse anti-rabbit and anti-mouse IgG HRP-linked from Amersham Biosciences; rabbit anti-VE-Cadherin from Santa Cruz Biotechnology. Fluorescein-conjugate rabbit anti-mouse IgG from Calbiochem. All the chemical products for protein extraction, electrophoresis, zymography and organ chamber were obtained from Sigma-Aldrich.

**Statistics**

ANOVA test was used to analyze variance between groups. When possible, Student’s t-test was performed. Value of $p \leq 0.05$ was considered significant.

**Results**

**Effect of shear patterns on endothelial function**

We analyzed endothelium-dependent vasodilatation in response to bradykinin (BK) stimulation on arterial segments using an organ chamber (Radnoti), according to previous works done on pig carotid arteries.$^{35,36}$ Relaxation was compared between the control arterial group at 0 days and the cultured arterial groups exposed to different types of shear stress during three days of perfusion (figure 2). We first confirmed that bradykinin relaxation could be completely blocked by the co administration of $N^\omega$-nitro-L-arginine methyl ester (L-NAME),
demonstrating the involvement of NO (data not shown). Vasodilatation response to bradykinin (10^{-9} - 10^{-6} mol/L) decreased significantly in arteries exposed to oscillatory shear stress (0.3±3 dynes/cm²) after three days of perfusion (figure 2C, *p≤0.001). On the contrary, exposure to unidirectional shear stress (6+/−3 or 0.3+/−0.1 dynes/cm²) maintained endothelial function to levels comparable to those observed in control arteries (figure 2A, 2B). Relaxation response in reply to a single concentration of bradykinin (10^{-7} mol/L) further supported this conclusion (figure 2D, *p≤0.003). Differences in vasodilatation response to bradykinin were also statistically significant when compared between the groups exposed to different flow patterns, further demonstrating that oscillatory shear stress affected endothelial function in perfused arteries. Interestingly, after 1 day of perfusion, no differences were observed between groups, underlying that such a short period of perfusion was not sufficient to reveal the effect of flow pattern on endothelial function (data not shown).
Figure 2: Bradykinin (BK) dose-dependent vasodilatation in porcine carotid artery segments immediately after harvesting (control group at day 0) and after 3 days of exposure to different flow patterns. The different groups were exposed to A) high shear stress (6±3 dynes/cm²), B) low shear stress (0.3±0.1 dynes/cm²), and C) oscillatory shear stress (0.3±3 dynes/cm²). D) Quantitative analysis of carotid vasodilatation responses to 10⁻⁷ mol/L of BK after 3 days of perfusion. The bradykinin relaxation is compared with group of fresh arteries at 0 days. Data are expressed as mean± SD. *P ≤ 0.02 vs. control group.
Effect of shear stress patterns on eNOS protein expression

We first demonstrated that VE-cadherin expression, a specific marker of endothelial cell, was not affected by shear stress patterns (figure 3B), allowing us to use this protein to normalize eNOS protein level to the total amount of endothelial-derived proteins contained in each time sample. Consequently, eNOS protein levels was determined and normalized to the expression of VE-cadherin and β-actin (figure 3A). After 1 day of perfusion, a slight increase in eNOS protein expression was found in groups of arteries submitted to unidirectional shear stress as compared to the 0 day group (figure 3C). Consistent with the data of endothelial function evolution during perfusion, oscillatory shear stress clearly inhibited the expression of eNOS gene after three days of culture, as compared to the control group (figure 3D, *p ≤ 0.02). The decrease in eNOS protein level was statistically significant also when compared to arteries exposed to unidirectional high shear stress (figure 3D, p ≤ 0.02). On the other hand, unidirectional shear stress maintained eNOS protein expression at levels comparable to the control group.
Figure 3: Effect of shear stress perfusion on eNOS protein expression
A) Representative immunoblot showing expression eNOS (140 kDa), VE-cadherin (130 kDa), and β-actin (55 kDa), B) The ratio of VE-cadherin/β-actin is measured to confirm no influence of shear stress on VE-cadherin protein expression, C) eNOS protein expression was measured after one day, and D) after three days of ex-vivo perfusion. Data are expressed as mean± SD, * P ≤ 0.02 vs. control group.
Effect of shear stress pattern on smooth muscle cell contraction

In order to assess whether exposure to different shear stress patterns also affected SMC contractile function, vasoconstriction in response to different doses of norepinephrine (NE) (10^{-9} -10^{-6} \text{ mol/L}) was measured before and after perfusion. NE-induced contraction was significantly increased in cultured arterial segments exposed to flow patterns for three days as compared to control arteries at 0 days (figure 4A, B, C, \( *p \leq 0.02 \)). This effect is also illustrated in the bar graph showing the SMC dependent vasoconstriction to a defined amount of NE (10^{-6}\text{mol/L}) (figure 4D). No statistical difference in contraction capacity between the arteries exposed to unidirectional or oscillatory shear stress was observed.
Figure 4: Norepinephrine (NE) dose-dependent constriction in porcine carotid artery segments immediately after harvesting (control group at day 0) and after 3 days of exposure to different flow patterns. A) High shear stress (6±3 dynes/cm²), B) low shear stress (0.3±0.1 dynes/cm²), C) oscillatory shear stress (0.3±3 dynes/cm²). D) Quantitative analysis of carotid vasoconstriction responses to 10⁻⁵ mol/L of NE after 3 days of in vitro perfusion. NE-induced vasoconstriction is compared with the arterial group at 0 days. Data are expressed as mean±SD. * P≤ 0.02 vs. control group.
Effect of shear stress patterns on smooth muscle phenotype

To investigate the influence of shear stress on SMC phenotype, we assessed the expression of smoothelin B, a marker of SMC differentiation \(^{37}\), and Ki67, a marker of cellular proliferation \(^{38}\), on arterial segments at 0 days and arterial segments exposed during 3 days at unidirectional high, unidirectional low and oscillatory shear stress. Smoothelin B expression appeared to increase significantly in the oscillatory and unidirectional high shear stress groups when compared to the 0 days group (figure 5A, \(^{*}p<0.02\)). Ki67 expression did not change after 3 days of perfusion when compared to 0 days, proliferative activity of SMC was very low and similar in all flow treatment groups (figure 5C).
Figure 5: A) Effects of shear stress on smoothelin B protein expression in arteries immediately after harvesting (day 0) and after 3 days of exposure to unidirectional high, unidirectional low, and oscillatory shear stress. Data are expressed as mean± SD. * P≤ 0.02 vs. control group. B) Representative immunoblot showing smoothelin B protein bands (100 kDa) in arteries exposed to the three shear stress patterns. C) The proliferative activity of SMC was visualized through Ki67 immunostaining on cross sections of arteries exposed for 3 days to unidirectional high, unidirectional low, and oscillatory shear stress and compared to the control group at 0 days. The microscope shows the Ki67 reaction in green and elastin visualized in red. All images were taken at x20 original magnification.
Effect of shear stress on matrix metalloproteinase expression

We also analyzed metalloproteinase-2 and –9 (MMP-2 and MMP-9) expression and activation, in order to investigate whether exposure of arteries to unidirectional or oscillatory shear stress induced a vascular remodeling process. MMP-2 gene expression (figure 6A, *p<0.01) and its gelatinolytic activity (figure 6B, *p<0.001) increased after 3 days of in vitro perfusion when compared to control arteries, the increase being significant for all shear stress patterns applied. The MMP-2 expression levels for arteries exposed during 3 days to oscillatory shear stress were significantly higher than those of the unidirectional low shear stress group. MMP-9 expression showed an increase in arteries exposed to oscillatory shear stress as compared to the control arteries (*p<0.002), as well as to unidirectional shear stress groups (figure 6C, p<0.04). Analysis of immunostained sections confirmed the results obtained by immunoblotting. MMP-9 immunostaining was positive only in arteries exposed to oscillatory shear stress (figure 6D). Further, analysis of immunostained sections showed preferential expression of MMP-9 in the external media suggesting an enhanced secretion of this enzyme by smooth muscle cells in this area.
Figure 6: A) MMP-2 protein expression in carotid arteries subjected to the three shear stress patterns. B) MMP-2 activation rate analyzed by zymography was calculated as the ratio of active over total MMP-2. Data are expressed as mean± SD. * P ≤ 0.02 vs. control group. C) MMP-9 protein expression in carotid arteries subjected to the three shear stress patterns. Data are expressed as mean± SD. * P ≤ 0.02 vs. control group. D) MMP-9 distribution on cross section of an artery exposed during 3 days to oscillatory shear stress as compared to a control artery at 0 days. The micrograph shows elastin layers visualized in red, smooth muscle cell nuclei in blue, and MMP-9 immunopositive reaction in green. All images were taken at x20 original magnification and were scaled identically.
Discussion

In the present study we investigated the effect of shear stress patterns on endothelial and smooth muscle cell function and on endothelial nitric oxide synthase (eNOS) in arterial segments perfused in vitro.

In the recent years, the importance of hemodynamic forces on endothelial cell (EC) and smooth muscle cell (SMC) has become more and more evident. An ever-increasing volume of data suggests that the high unidirectional flow, typical of plaque-free areas, exerts a protective effect on EC whereas a cyclic reversal of flow direction combined with a low mean shear stress, as often observed at bifurcations and branching points, favors the development of atherosclerotic plaque. Several studies using isolated EC have demonstrated that EC exhibit a capacity to discern between different hemodynamic environments. Nevertheless, the way hemodynamics predispose or protect against plaque development are not fully understood. Further, the vast majority of studies on the effects of hemodynamics on EC function were performed on cell cultures using appropriate devices for applying physiologically relevant mechanical forces. Cells in culture, however, lack the cell-matrix as well as the EC-SMC interactions, which may be essential in determining the cell response to hemodynamic force solicitations, especially at long-term. To overcome the inherent limitations of cell studies, we investigated the effects of hemodynamics on EC and SMC on arterial segments using an ex-vivo arterial support system (EVASS), previously described by Bardy et al.

One of the main findings reported in this study is that unidirectional shear stress maintained eNOS protein level expression and preserved endothelial-derived vasorelaxation. These effects were independent of the level of mean shear stress value as well as the level of pulsatility in flow. On the contrary, in pig arteries exposed to reversal oscillatory shear stress, eNOS protein level and endothelial function were down regulated. These differences in endothelial function did not influence contractile capacity of medial SMC. Indeed, in all groups analyzed, an improvement of SMC function was observed as compared to fresh arteries, indirectly proving that our perfusion system is performing well for arterial culture.
In vitro studies using isolated endothelial cells have shown that after 24 hours of unidirectional shear stress, eNOS gene expression was up-regulated, whereas oscillatory shear stress failed to induced such expression.\textsuperscript{20,22,41} In concordance to previous works, a decrease in eNOS protein level was observed after three days of exposure to oscillatory shear stress (figure 3D). No decrease has been noticed after one day of perfusion (figure 3C). The discrepancies in reaction time observed in relation to the previous work using isolated cells may be attributed to different composition of basal extracellular matrix influencing EC response. Intragroup variations in eNOS expression level tend to decrease after three days of perfusion as compared to arteries perfused for one day only (figure 3C,D). This observation demonstrates that, after a period of three days, arteries adapt and become more homogenous in their response to the new hemodynamic environment.

Previous studies investigating the NO synthesis in cultured EC have illustrated that the dynamic characteristics of flow modulate the production of vasoactive mediators, such as NO and endothelin-1 (ET-1).\textsuperscript{21,43} In our study, we analyzed the evolution of medial and endothelial function using an organ chamber system (Radnoti) before and after exposure to different hemodynamic conditions. A significant decrease of endothelial function was observed on arteries exposed for three days to an oscillatory shear stress as compared to the control arteries at 0 day (figure 2C). This relevant decrease of endothelial-derived vasodilatation occurred due to a decrease of NO production. Indeed, in our experimental conditions, bradykinin-induced vasorelaxation has been demonstrated to be completely abrogated by the concomitant use of the L-arginine analog L-NAME (data not shown). The unidirectional high and low shear stress did not affect endothelial-derived vasorelaxation (figure 2 A, B). These data further demonstrate that plaque-prone flow directly modulates endothelial function by impairing NO production.

Giraldez et al. showed that endothelial dysfunction in isolated hearts perfused in vitro, does not require loss of eNOS but can be triggered by loss of eNOS substrate or cofactors.\textsuperscript{44} Our experiments showed that a clear correlation between decreased eNOS synthesis and decreased endothelial function exists in arteries exposed to an oscillatory flow, and have demonstrated that the amount of eNOS protein is a limiting factor for the endothelial function.\textsuperscript{25} This apparent paradox could be explained by the difference in vascular vs. cardiac endothelium and by differences
in the experimental design. Nevertheless, all these data point out the complexity of NO metabolism. Although the decrease of NO production in arteries exposed to oscillatory flow induced a decrease of endothelium-derived vasorelaxation, impaired NO synthesis had no influence on SMC function (figure 4). After three days of exposure to different flow patterns, the NE-derived SMC contraction was improved as compared to control arteries. These results are also confirmed by the expression of smoothelin B, a differentiation-marker for SMC\(^\text{45}\) of the contractile phenotype. Smoothelin B increased after 3 days of exposure to oscillatory and unidirectional high shear stress as compared to control arteries at 0 days (figure 5A).

NO has been shown to have an antiproliferative effect on SMC.\(^\text{43}\) Previous studies reported that alterations in shear stress affected the accumulation of medial tissue mass and increased the proliferation of SMC.\(^\text{46-48}\) In our studies the proliferative activity of SMC as visualized through the Ki67 immunostaining, was not affected by the different flow patterns (figure 5C). These data may be explained by the fact that the lowered endothelial-derived NO synthesis, observed in arteries exposed to oscillatory flow was still sufficient to preserve medial wall metabolism, or that consequences at the level of SMC need a longer time to appear.

Chronic exposure of the arterial wall to oscillatory shear stress has been associated with structural remodeling\(^\text{49,50}\) whereas a sudden change in blood flow and wall shear stress induces an increased expression of MMPs.\(^\text{51}\) Arterial remodeling in response to flow and wall shear stress alterations is endothelial dependent.\(^\text{50,52}\) Indeed, first the endothelial cells and later SMC express MMPs in response to flow changes, suggesting that both cell types contribute to flow-induced remodeling.\(^\text{53,54}\) In the present work, three days of exposure to shear stress resulted in an increased expression and activation of MMP-2 when compared to control arteries at 0 days (figure 6A,B). Exposure to oscillatory shear stress induced an increased expression of MMP-9 when compared to the other flow treatments. These observations are consistent with previous works reporting that MMP-9 expression is up-regulated in response to oscillatory shear stress.\(^\text{55}\) The cited study investigated the response of isolated endothelial cells in culture. Our results do not allow identification of cellular origin of secreted MMP-9. Nevertheless, MMP-9 immunostaining in arteries exposed to oscillatory shear stress, was present only in the media, suggesting that SMC are
the main contributors. Our observations, however, do not exclude a role of EC in this process.

In conclusion, oscillatory shear stress with flow reversal, mimicking plaque-prone conditions, leads to local impairment of NO production by endothelium, resulting from a decrease of eNOS enzyme synthesis. These decreased endothelial function did not influence smooth muscle cell function for the length of the perfusion time considered. These data further support the hypothesis that unidirectional shear stress prevents atherosclerotic plaque development by means of preserving high level of eNOS expression and consequently good endothelial function. Our study is the first to report a model on the effects of well-defined oscillatory shear stress on isolated arterial segments, preserving thus the possible effects of vascular cell interactions as well as cell-extracellular matrix interactions. The present work constitutes a novel approach for the study of flow-induced arterial function and remodeling and proposes a valuable tool in the investigation of the role of hemodynamics in the initiation of arterial disease.

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**References**


Although a growing body of work contributes to the understanding of blood vessel remodeling, there is still a need to identify the accurate links existing between the biological events and the biomechanical stimuli implicated in the remodeling response. The limitations inherent to several of the studies proposed have been mainly linked to the methodological approach. The two prevailing methodological tendencies have been at one end cellular in vitro studies where cells are isolated from their native immediate environment and deprived of the related biochemical interactions, and at the other end in vivo studies where systemic factors affect the remodeling response, hence influencing the interpretation of the events. The approach to the study of vascular remodeling developed in this thesis is based on a new model of organ in vitro culture. The proposed model allows the dissociation and control of the various mechanical factors acting on the arterial wall and their individual analysis in relation to the biological response involved in the adaptive process. This approach takes into account the interaction between vascular cells and the extracellular matrix in the adaptation to hemodynamic changes.

Through the present work we provide experimental evidence on various aspects of wall remodeling and in particular on modification of wall properties and structure in response to a change in the external and internal hemodynamic environments.

The in vitro adaptive response of the porcine carotid artery, which is characterized by a non homogenous and non-uniform wall, entails the reorganization and turnover of the main structural proteins, elastin and collagen, and seems to be mediated by the degradative action of MMP-2. The resulting remodeled wall shows uniform scleroprotein distribution and is more distensible at physiological pressures.

Arterial adaptation to steady pressure entails an elastolytic process of the arterial wall mediated by MMP-2 and leading to increased distensibility. In contrast, exposure to pulsatile pressure appears to reduce matrix degradation and increase wall stiffness by augmenting the relative elastin content.

Arterial adaptation to shear stress variations is endothelium dependent and is transiently mediated by nitric oxide (NO) metabolism. In normal arteries, flow direction appears to be a determinant factor influencing
both endothelial function and NO production. Exposure to oscillatory shear stress with flow reversal of the heart cycle, mimicking pathology-prone conditions, decreases endothelial function and alters NO metabolism by decreasing the synthesis of eNOS, whereas unidirectional shear stress has a protective effect in maintaining the function of endothelial cells. Flow conditions do not seem to alter the contractile function of SMC. Remodeling of the arterial wall exposed to oscillatory flow appears to be due to increased expression of MMP-2 and MMP-9.

In normal arteries the structural changes, the biomechanical response and the enzymatic implications are intimately linked to the remodeling process. Adaptations to changes in tensile stress mostly affect the media and MMP-2 seems to be the main key actor implicated in extracellular matrix reorganization and turnover. Adaptations to changes in shear stress are dependent on flow pattern and primarily affect the intimal layer of the arterial wall, resulting in changes of endothelial function and NO metabolism. MMP-2 and MMP-9 appear to be the major agents implicated in extracellular matrix reorganization and turnover in flow-induced remodeling.
The model developed in this study constitutes a valuable tool for the study of vascular remodeling through approaches yet unexplored. The effects of mechanical factors on arterial adaptation may be further studied by combining tensile stress and shear stress variations. Creating such a complex hemodynamic environment favoring intimal formation and loss of endothelial function may help to identify new mechanisms involved in the regulation of arterial wall structure and function and their relation to disease. Deeper research on pathophysiological process related to vascular remodeling may be performed by mimicking an inflammatory in vitro environment (e.g. through the addition of macrophages or interleukins into the culture system).

Further research should point to elucidate the events involved on the conversion of mechanical stresses into chemical signals (mechanotransduction), the triggering of intracellular signaling pathways and the mechanisms of regulation of the adaptive response. Of special interest is the hypothesis that regulation of MMPs could inhibit the progress of various cardiovascular diseases, thus preventing their clinical consequences. Such approaches prospect new therapeutic alternatives relying on the development of specific inhibitors, appropriate animal models and new diagnostic tools.
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